Pressure Denaturation of the Bacteriophage P22 Coat Protein and Its Entropic Stabilization in Icosahedral Shells

Peter E. Prevelige Jr.,* Jonathan King,‡ and Jerson L. Silva§

*Boston Biomedical Research Institute, Boston Massachusetts 02114 USA; ‡Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 USA; §Departamento de Bioquimica, Instituto de Ciencias Biomedicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941, Brazil

ABSTRACT The pressure stability of bacteriophage P22 coat protein in both monomeric and polymeric forms under hydrostatic pressure was examined using light scattering, fluorescence emission, polarization, and lifetime methodology. The monomeric protein is very unstable toward pressure and undergoes significant structural changes at pressures as low as 0.5 kbar. These structural changes ultimately lead to denaturation of the subunit. Comparison of the protein denatured by pressure to that in guanidine hydrochloride suggests that pressure results in partial unfolding, perhaps by a domain mechanism. Fluorescence lifetime measurements indicate that at atmospheric pressure the local environments of the tryptophans are remarkably similar, suggesting they may be clustered. In contrast to the monomeric protein subunit, the protein when polymerized into procapsid shells is very stable to applied pressure and does not dissociate with pressure up to 2.5 kbar. However, under applied pressure the procapsid shells are cold-labile, suggesting they are entropically stabilized. The significance of these results in terms of virus assembly are discussed.

INTRODUCTION

The coat protein subunits comprising the capsids of spherical viruses with T > 1 associate through the formation of nonequivalent bonding interactions (Caspar and Klug, 1962; Rossmann, 1984). Crystallographic studies have demonstrated that these nonequivalent interactions are realized by conformational changes within the protein subunits (reviewed in Rossmann and Johnson, 1989). Thus, chemically identical protein subunits take up unique positionally dependent conformations during the process of assembly. The mechanism by which the plasticity required for successful assembly is coded into the folded conformation of the monomeric protein subunit is fundamental to the understanding of the viral assembly process.

The polymerization of protein subunits into large oligomeric structures, such as viruses, is generally accompanied by an increase in system volume (Heremans, 1982; Weber and Drickamer, 1983). This somewhat counterintuitive result is due to the combined effects of the formation of solvent excluding cavities at the intersubunit interfaces and the release of bound solvent (Silva and Weber, 1993). Solvent release can increase interatomic distances (and thereby system volume) by two mechanisms: the burying of nonpolar amino acid residues is accompanied by replacement of dipole-induced dipole bonds (water-amino acid interactions) with London dispersion forces between the amino acids in the interface and through the formation of intersubunit salt linkages. Since the system volume increases upon polymerization, it is frequently possible to dissociate protein polymers by the application of pressure. Dissociation of the polymer to its constituent subunits frequently occurs in the pressure range from 1.0 to 2.5 kbar (Heremans, 1982; Weber and Drickamer, 1983; Silva and Weber, 1993), where the protein subunits themselves generally do not denature. The effect of temperature on protein conformation is often explored to derive thermodynamic information about protein stability. However, it is expected that the effects of pressure will be equally informative and perhaps more readily interpretable (reviewed in Silva and Weber, 1993).

To determine the effect of pressure on the conformation of a viral coat protein, we have undertaken to study the effect of pressure on polymerized and monomeric forms of the coat protein of the *Salmonella* bacteriophage P22. This system has the advantage that biologically active monomeric coat protein can be prepared readily, along with the polymerized form of the capsid. When mixed together in solution with the scaffolding protein the subunits rapidly polymerize and kinetic and biochemical analysis has revealed details of the assembly pathway (Prevelige et al., 1988, 1993). Furthermore, this protein is remarkable in that the polymerized form undergoes remarkable changes in morphology triggered by DNA packaging (Prasad et al., 1993).

Bacteriophage P22 is a double-stranded DNA containing bacteriophage. Its morphogenetic pathway requires the formation of a precursor procapsid which subsequently matures to the capsid. The procapsid is composed of 420 molecules of coat protein arranged around an inner core composed of approximately 300 molecules of scaffolding protein (King and Casjens, 1974). The phage DNA is packaged into this preformed procapsid through a portal protein complex located at a single vertex (Bazinet et al., 1988). As the DNA is packaged, the scaffolding protein exits and recycles to participate in further rounds of assembly. It is possible to extract the scaffolding protein from the procapsid by mild

Received for publication 29 September 1993 and in final form 4 February 1994.

Address reprint requests to Dr. Peter E. Prevelige, Boston Biomedical Research Institute, 20 Staniford St., Boston MA 02114. Tel.: 617-742-2010; Fax: 617-523-6649; E-mail: prevelige@bbri.eri.harvard.edu.

0006-3495/94/05/1631/11 $2.00

treatment with GuHCl (Fuller and King, 1981). This process leaves intact a shell of coat protein. A three- dimensional reconstruction of the protein lattice of both the extracted procapsid and the capsid has recently become available (Prasad et al., 1993). The procapsid has an overall spherical shape, with an outer diameter of 580 Å. The coat protein subunits are arranged on a T = 7 lattice, and within the asymmetric unit of the lattice the subunit disposition in the procapsid is markedly asymmetric, suggesting substantial differences in bonding interactions among the subunits. In the capsid which has packaged DNA the subunits remain arranged on a T = 7 lattice, the subunits undergo substantial conformational changes resulting in an overall expansion of the lattice, closure of holes in the lattice, and the formation of new intersubunit contacts (Prasad et al., 1993). This transition appears to be exothermic in nature (Galisteo and King, 1993), and it has been suggested that the energy derived from this process may play a role in DNA packaging (Steven, 1993). Raman spectroscopy suggests that the differences between both successful subunit assembly into the procapsid and procapsid expansion involve domain movement with very little change in secondary structure (Prevelige et al., 1993).

The results described in this paper suggest that the monomer of coat protein is only marginally stable when unpolymerized and that it derives substantial energy of stabilization through the contacts within the lattice. Furthermore, we have shown that the driving force for subunit assembly is entropic in nature.

MATERIALS AND METHODS

Preparation of protein samples

The preparation of procapsids, empty shells, and coat protein monomers was as previously described (Prevelige et al., 1988). The procapsids employed were purified from *Salmonella typhimurium* strain DB7136 which had been infected with P22 phage carrying 2^- am/13^- am mutations. The 2^- mutation blocks DNA packaging resulting in the accumulation of procapsids, while the 13^- delays lysis, thereby increasing the yield.

The coat protein monomer was stored as a 1.4–1.8 mg/ml stock solution in 2 M GuHCl and dialyzed overnight at 4°C against 50 mM Tris, 25 mM NaCl, 2 mM EDTA pH 7.6 in Spectrapor 2 dialysis tubing prepared as previously described (Prevelige and Fasman, 1983). The absorption spectra of the proteins were recorded immediately before use both to determine the concentration and as a check for light scattering. In the case of the protein prepared at 1.0 mg/ml, the dialyzed sample was centrifuged in a Beckman TL-100 centrifuge equipped with a TL-100.3 rotor at 48,000 rpm for 20 min.

Coat protein at 0.2 mg/ml in 5.5 M GuHCl was prepared by dilution of the stock solution in 2 M GuHCl into 6 M GuHCl prepared in 50 mM Tris, 25 mM NaCl, 2 mM EDTA pH 7.6. The diluted protein was dialyzed as described above.

Optical methods

Fluorescence spectra

In experiments where the pressure was altered, the pressure was changed and then the sample allowed to equilibrate for 5 min before making measurements. To determine whether the sample was at practical equilibrium, we recorded sequential spectra without pressure changes at both the high and low pressure limits. There was no time-dependent change in the fluorescence properties.

The high-pressure bomb, purchased from SLM-Aminco (Urbana, IL) has been described by Paladini and Weber (1981). Fluorescence spectra were recorded on a ISS 200 computer-controlled spectrofluorometer (Champaign, IL).

Fluorescence spectra at pressure p were quantified by specifying the center of spectral mass $\langle \upsilon_p \rangle$.

$$\langle \upsilon_p \rangle = \sum_i \upsilon_i \cdot F_i \Big/ \sum_i F_i \tag{1}$$

where F_i stands for the fluorescence emitted at wave number υ_i and the summation is carried out over the range of appreciable values of F. The degree of dissociation (α_p) is related to $\langle \upsilon_p \rangle$ by the expression:

$$\alpha_p = \left(\frac{1 + Q(\langle \upsilon_p \rangle - \langle \upsilon_f \rangle)}{\langle \upsilon_i - \langle \upsilon_p \rangle^{-1}} \right)^{-1} \tag{2}$$

where Q is the ratio of the quantum yields of dissociated and associated forms, $\langle \upsilon_p \rangle$ is the center of spectral mass at pressure p, and $\langle \upsilon_f \rangle$ and $\langle \upsilon_i \rangle$ are the corresponding quantities for dissociated and associated forms (Paladini and Weber, 1981; Silva et al., 1986; Royer et al., 1986; Silva and Weber, 1988).

Fluorescence anisotropy

Fluorescence anisotropy measurements were made in a L-format polarization fluorescence. The corrections for the scrambling of the windows were performed as described by Paladini and Weber (1981).

Tryptophan lifetime and rotation measurements

Lifetime and dynamic depolarization measurements were performed by a multifrequency cross-correlation phase and modulation fluorometer which uses the harmonic content of a high-repetition-rate, mode-locked Nd-YAG laser. This laser is used to synchronously pump a dye laser whose pulse train is frequency doubled with an angle-tuned frequency doubler (Alcala et al., 1985). A detailed description of phase fluorometry lifetime measurements and data analysis has been fully described previously (Gratton et al., 1984; Lakowicz et al., 1984; Beechem et al., 1991). The quality of fits was assessed by χ^2 values and by plots of weighed residuals. Excitation wavelength was 295 nm and the emission was observed through a long-wavelength pass filter (WG 335) with a cutoff at 335 nm. For the lifetime studies at atmospheric and high pressure, light scattering of the sample at 295 nm (interference filter from Corion) was used as reference. The same results were obtained when the measurements were performed at atmospheric pressure inside the pressure bomb or in a regular cuvette and utilizing p-terphenyl in cyclohexane in the reference cell.

Light scattering

Light scattering measurements were made in an ISS 200 spectrofluorometer (Silva et al., 1989). Scattered light (320 nm) was collected at an angle of 90° of the incident light.

HPLC methods

HPLC was carried out in a Waters system. A prepackaged SynChropak GPC500 column (250 × 4.6 mm inner diameter), obtained from SynChropak, Inc. (Linden, IN), was utilized for the gel filtration of procapsid shells and a TSK 3000 column was utilized for the gel filtration of the coat protein. The system was equilibrated in Tris (50 mM)–sodium acetate (0.2 M) buffer (pH 7.0), and sodium azide (0.5 g/l). A flow rate of 0.3 ml/min was utilized. Sample elution was monitored by absorption at 280 nm. The column void volume (V_o) and total volume (V_t) were determined with lambda phage DNA and ADP, respectively.

RESULTS

HPLC characterization of the coat protein

The monomeric coat protein in Tris buffer and the shells of coat protein were analyzed by size exclusion HPLC before spectral analysis (Fig. 1, *A* and *B*). The coat protein monomer eluted in primarily as a single peak with an apparent molecular weight of ~60,000 M_r based on globular protein standards. This is in accord with the behavior previously described (Fuller and King, 1981; Prevelige et al., 1988). The small leading peak probably represents aggregated coat protein. The shells also eluted in a single peak within the included volume of the column. The results show that the shells do not contain significant quantities of coat protein monomer, and likewise the monomers do not contain significant amounts of polymerized forms of coat protein.

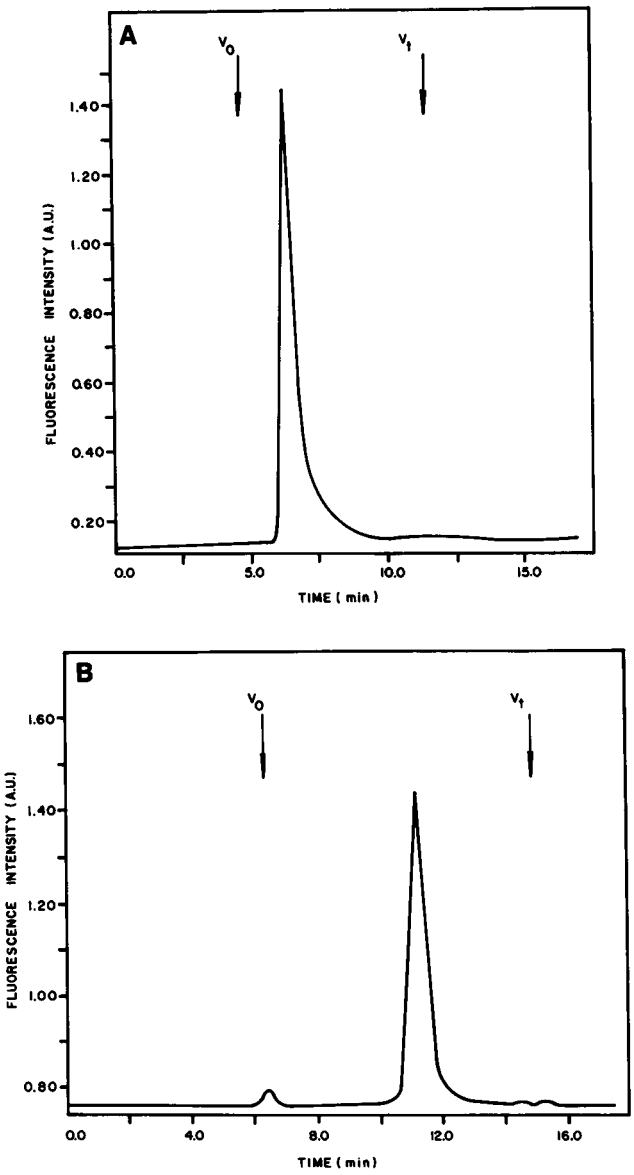

FIGURE 1 Size exclusion high performance liquid chromatography of P22 coat protein and procapsid shells. Procapsid shells were run on a GPC500 column (*A*) and refolded coat protein was run on a TSK 3000 column (*B*) as described in Materials and Methods.

Steady-state fluorescence spectra of coat protein

The coat protein contains six tryptophan residues distributed more or less uniformly through the primary sequence (Eppler et al., 1991). The fluorescence spectra of empty shells of coat protein and the spectra of monomeric coat protein were recorded (Fig. 2 *A*). The spectrum of the empty procapsid shell shows a small blue shift relative to that of the folded monomer, indicating that the tryptophans are less solvent exposed in the polymerized form in accord with the observations by Teschke and King (1993).

Upon denaturation of the monomer with GuHCl there is a substantial red shift in the tryptophan fluorescence (Fig. 2 *B*). A similar change was observed when the monomeric protein was examined under a pressure of 2.6 kbar, suggesting that pressure caused denaturation. The following centers of spectral mass in wave numbers were determined from the recorded spectra: shell (29,226 cm^{-1}), folded monomer (28,980 cm^{-1}), GuHCl denatured reference state (28,280 cm^{-1}), protein under pressure (28,450 cm^{-1}). These changes in spectral mass, along with changes in light scat-

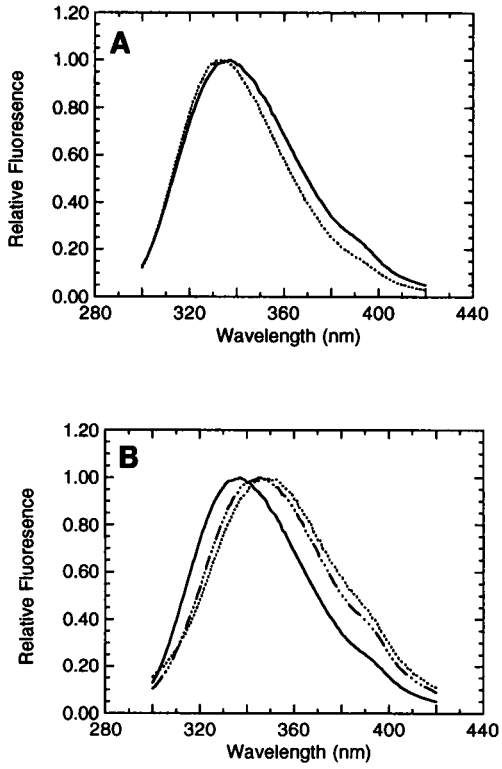

FIGURE 2 (*A*) Tryptophan fluorescence emission of P22 procapsid shells (· · ·) and native P22 coat protein monomers (———). (*B*) Tryptophan fluorescence emission of native P22 coat protein monomers (———), guanidine-denatured (· · ·), and pressure-denatured coat proteins (— · · · —). Spectra were recorded on an ISS 200 spectrofluorometer. The excitation wavelength was 280 nm, and the excitation and emission bandwidths were 1 and 1 mm, respectively.

tering were employed to follow the effects of pressure upon subunit conformation.

The pressure stability of coat protein monomers

The stability of purified coat protein monomer at 0.2 mg/ml to pressure at 1°C and 20°C was examined (Fig. 3). At both temperatures the pressure was varied from atmospheric to 2.6 kbar. In one experiment both fluorescence spectra and light scattering were recorded and in a second experiment only the fluorescence spectra were recorded. At neither temperature was there significant change in the level of light scattering during the course of the pressure cycle as would be expected if there was significant aggregation.

At both temperatures as the pressure was increased the fluorescence spectrum underwent a red shift (the center of spectral mass decreased) indicative of increased solvent exposure. This shift appeared to occur in two transitions as monitored by fluorescence. At 20°C the first relatively sharp transition occurred at a very low pressure, with a midpoint of approximately 0.25 kbar (Fig. 3 *A*). The second more gradual transition occurred over a higher pressure range. Two transitions were also reported in the renaturation curve of the protein from GuHCl (Teschke and King, 1993).

At the lower temperature (0°C) (Fig. 3 *B*), the first transition appeared more cooperative than it did at 20°C. Although the same final center of spectral mass was obtained, the relative contributions of the two transitions to the total change also appeared to be temperature-dependent. At low temperature a higher fraction of the total change is associated with the first transition. The pressure at which one-half of the total change in the center of spectral mass is obtained is ~0.45 kbar at 1°C and 0.65 kbar at 20°C. This suggests that decreasing temperature destabilizes the structure of the monomer toward pressure.

Upon returning to atmospheric pressure, both samples regained some of the original spectral characteristics, suggesting that the process may be partially reversible (Fig. 3, *A* and *B*). The changes induced in the high pressure range were more reversible than those induced in the low pressure range.

If the pressure-induced transition represented a unimolecular process such as denaturation, the pressure required to induce the transition should be independent of protein concentration. If it represented a transition involving the dissociation of an oligomer, it would be expected to display a concentration dependence. Therefore, we repeated the experiment described above at a concentration of 1.0 mg/ml at 20°C (Fig. 3 *C*). There was no significant effect of protein concentration on the pressure midpoints of the transitions. Once again, the transition as monitored by fluorescence appeared to be at least partially reversible.

To determine whether the structural transitions induced by pressure were in fact reversible, we examined the ability of the coat protein to assemble into procapsids in the presence of added scaffolding protein both before and after pressure treatment. In both cases the subunits were competent for assembly, although the rate of assembly with the pressure cycled coat protein was somewhat decreased (data not shown). The decrease in the rate of assembly is consistent with the presence of some inactive protein.

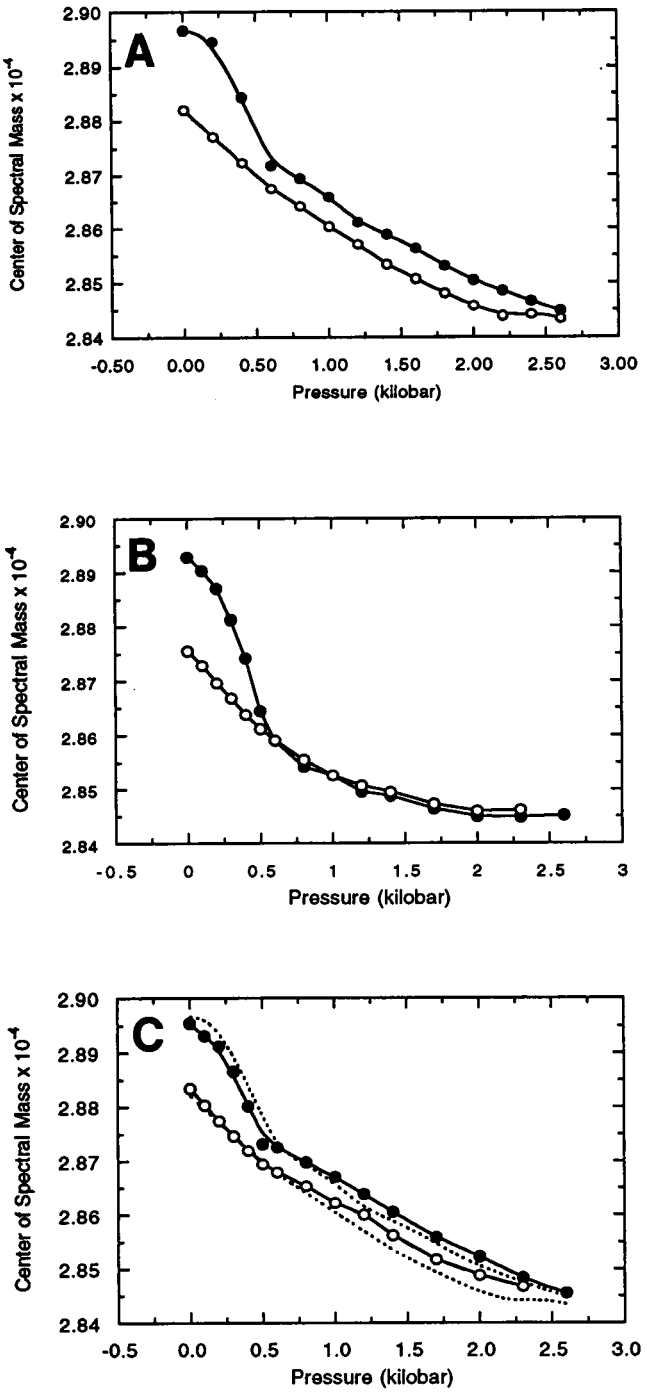

FIGURE 3 Pressure denaturation of P22 coat protein monomers. (*A*) Plot of center of spectral mass vs. pressure at 0.2 mg/ml coat protein, 20°C. (*B*) Plot of center of spectral mass vs. pressure at 0.2 mg/ml, 1°C. (*C*) Plot of center of spectral mass vs. pressure at 1 mg/ml, 20°C. In all cases the closed circles represent increasing pressure, while the open circles represent the return to atmospheric pressure. The dotted line in (*C*) represents the transition observed at 0.2 mg/ml 20°C for comparison.

A likely cause of irreversibility was aggregation of the protein upon denaturation under pressure. To determine

whether the protein was aggregating, the sample was analyzed by HPLC after pressure cycling. The bulk of the protein eluted in the same position as the starting material; however, the size of the leading peak, assumed to be aggregate, was increased after pressure cycling (data not shown).

Since the pressure-induced unfolding appears to consist of two transitions, it was possible that the aggregation accompanied only the one occurring at the higher pressure. To test this hypothesis, we pressure cycled coat protein at 0.2 mg/ml and 20°C up to 1.05 kbar and back to atmospheric as rapidly as possible. In this case, although there appeared to be a slight increase in the amount of reversibility, the denaturation was still not wholly reversible.

Polarization measurements

The two transitions in the pressure-induced denaturation suggested that the protein might unfold with distinct domains. To obtain information about the hydrodynamic properties of the protein during the pressure-induced unfolding, we followed the transition by fluorescence polarization using intrinsic tryptophan fluorescence (Fig. 4). The steady-state polarization for the monomer of capsid protein was 0.208. This relatively high value suggests that tryptophan residues are mostly immobilized and that the polarization value reflects the rotation motion of the whole protein. Under application of pressure, there was a dramatic decrease in the polarization, apparently in a unimodal curve (Fig. 4 *A*). The final value of polarization of pressure-denatured coat protein was 0.105, close but not equal to that of guanidine-unfolded coat protein (0.085).

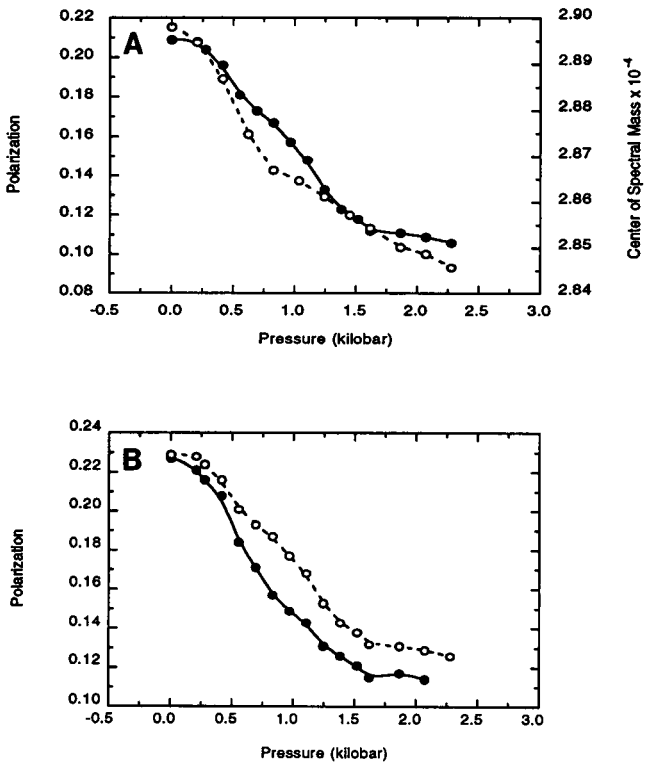

FIGURE 4 Effect of pressure on the tryptophan fluorescence polarization of P22 coat protein. (*A*) Polarization (●) and center of spectral mass (○) of 0.2 mg/ml solution of coat protein at 20°C. (*B*) Polarization of coat protein at 0.2 mg/ml at 20°C (●) and 1°C (○). Notice that the scales have been shifted by 0.02 units. This is the difference in polarization due increased solvent viscosity at decreased temperature.

The absence of two phases in the changes in polarization is discrepant with the fluorescence emission data that present two transitions. Fig. 4 *A* also compares the polarization and the fluorescence spectra changes. The decrease in polarization occur in a pressure range that corresponds to both phases of the spectral changes. It can be concluded that each phase of the spectral changes is followed by about the same amplitude in the changes of tryptophan rotations.

As was the case when the transition was followed by center of spectral mass, denaturation occurred at a lower pressure when the temperature was decreased to 1°C. Because of the lower temperature, both the polarization of the native protein as well as the one from pressure-denatured protein were about 0.02 units higher (Fig. 4 *B*). After decompression, the value of polarization returned to 0.213 (20°C) and to 0.223 (1°C), indicating that recovery of the rotations was almost completely reversible.

Lifetime of the excited state of coat protein tryptophan fluorescence

The excited-state lifetime of the tryptophanyl residues in coat protein was measured for the native, guanidine-denatured and pressure-denatured states (Fig. 5). The lifetime determinations were performed by multifrequency phase and modulation fluorometry using the harmonic content of a mode-locked laser (Alcala et al., 1985; Silva et al., 1992). For the native state, the best fit to the data was obtained using two discreet lifetime exponentials (Fig. 6 *A*; Table 1). This decay is rather unusual for a protein containing six tryptophans. Even for single tryptophan proteins, the decay is usually very complex (Beechem and Brand, 1985; Silva et al., 1992) and fitting requires a lifetime distribution approach (Alcala et al., 1987a,b) rather than a sum of exponentials. The high steady-state polarization observed suggested that during the lifetime of the excited state of the tryptophans, they do not exchange between different environments, and this constancy of environment may be a contribute to the unusually homogeneous decay. Since there are six tryptophans with similar environments, a reasonable possibility is that the tryptophans may occur in clusters within the protein. This would reduce the number of environments seen by the six tryptophan side chains. An alternative possibility is that there are closely spaced lifetimes which could not be discriminated. In fact, it has been shown that the four or five lifetime decays of the two tryptophan residues in lac (Royer et al., 1990) or trp (Royer, 1992) repressors collapse to only two in the wild-type proteins.

The addition of 5.5 M guanidine shifted the phase and modulation curves to higher frequencies (Fig. 5 *A*), and the data could be fit to a double-exponential decay (Fig. 6 *B*). The lifetime values in this case were shorter than those de-

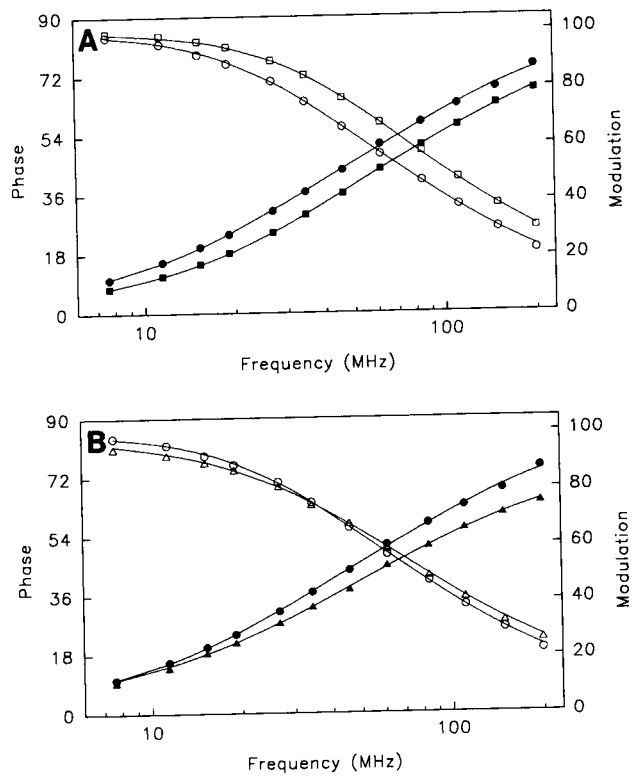

FIGURE 5 Tryptophan lifetime measurements of the native, guanidine-denatured, and pressure-denatured states of coat protein. (*A*) Phase (*solid symbols*) and modulation (*open symbols*) in the frequency range of 2–300 MHz were measured in the absence (*circles*) and in the presence (*squares*) of 5.5 M guanidine. (*B*) Phase (*solid symbols*) and modulation (*open symbols*) in the frequency range of 2–300 MHz were measured at atmospheric pressure (*circles*) and at 2.5 kbar (*triangles*). The lines correspond to the best fit for each set of data, using two discreet exponential for the native and guanidine- denatured coat proteins and a single-component Lorentzian distribution for the pressure-denatured coat protein. Excitation was at 295 nm and emission was observed through a WG 320 filter. Protein concentration was 0.2 mg/ml.

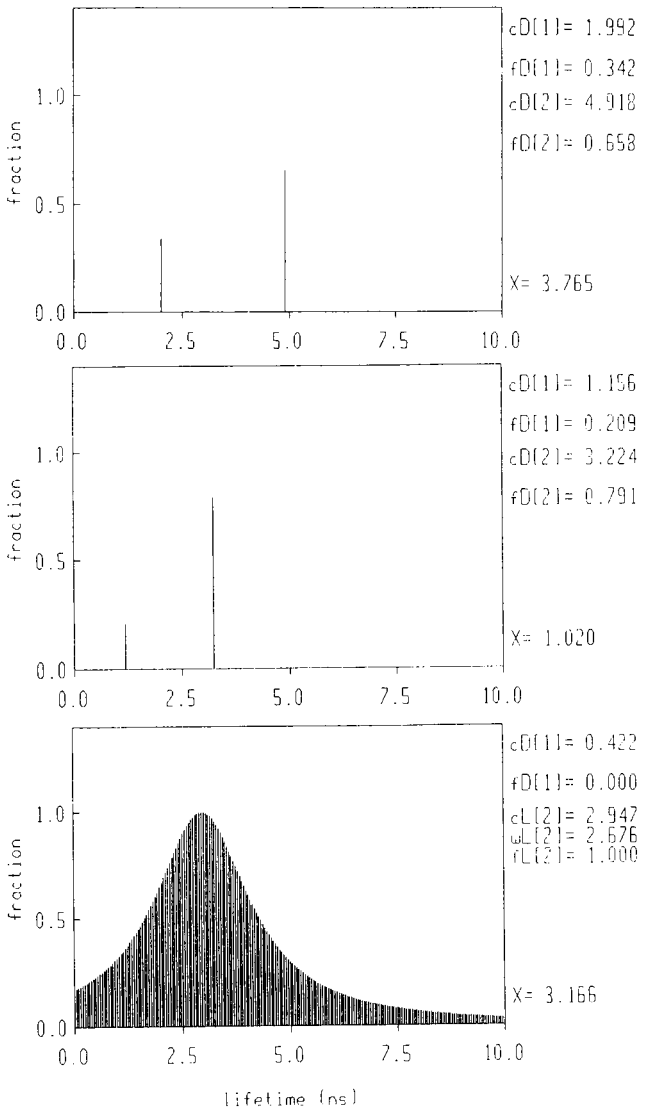

FIGURE 6 Fitting of lifetime data. (*A*) Atmospheric pressure; (*B*) 5.5 M GuHCl; (*C*) 2.2 kbar. The best fit corresponds to two discreet exponential for the native and guanidine-denatured coat proteins and a single-component Lorentzian distribution for the pressure-denatured coat protein.

termined for the native protein, as expected for solvent-exposed tryptophan residues.

Having measured the lifetimes in both the native and GuHCl- denatured reference states, we measured them in the pressure-denatured state. Fig. 5 *B* shows the phase and modulation data for the coat protein maintained at 2.4 kbar pressure. Similar to the case of the guanidine-denatured form, the phase angles shifted to higher frequencies. However, the demodulation in the pressure-denatured state was only slightly shifted to higher frequencies. Fitting the data to a sum of exponential was unsuccessful, resulting in large χ^2 (Table 1). The data could be satisfactorily fit to a Lorentzian distribution of lifetimes (Fig. 6 *C*). These data suggest that the conformation of the pressure-denatured state is different from the guanidine-denatured state. The increased breadth of the distribution might be indicative of presence of a complex equilibrium among different intermediate conformations under pressure, whereas in the presence of guanidine, the tryptophan residues experience more monotonous environments during the lifetime of the excited state.

Measurement of rotation diffusion by dynamic depolarization of tryptophan fluorescence

The rotation of the tryptophan residues in coat protein was measured using the pulsed-laser multifrequency phase-fluorometer (Gratton et al., 1986, Silva et al., 1992). Fig. 7 shows the differential phase and modulation data for coat protein at atmospheric pressure (Fig. 7 *A*), in the presence of guanidine (Fig. 7 *B*), and under pressure (Fig. 7 *C*). Least-squares analysis of the data was performed as described by Gratton et al. (1986). The best fit to the data for the monomer at atmospheric pressure was obtained using a model that assigns two rotational motions (Table 2). The slower motion (rotational correlation time τ_{c1} = 25 ns) is the predominant (f_1 = 0.56) and corresponds to the rotation of the whole protein. This value complies with that expected for the whole spherical rotation of a globular protein of about 65,000 M_r.

TABLE 1 Discrete exponential and continuous distribution analysis for lifetime data obtained for capsid protein

Capsid protein*	Two exponentials					One exponential and one distribution‡					
	τ1	f1	τ2	f2	χ^2	τ1	f1	c2	w2	f2	χ^2
A	2.01	0.35	4.97	0.65	4.1	2.00	0.33	4.85	0.19	0.67	4.3
G	1.15	0.21	3.21	0.79	1.3	1.17	0.21	3.22	0.05	0.79	1.5
P	1.30	0.35	5.01	0.65	32.8	0	0.004	2.99	2.62	0.996	3.6

* A, native; G, 5.5 M guanidine-denatured; P, pressure-denatured.
‡ c, center; w, width; f, fractional intensity of the Lorentzian lifetime distribution.

The second rotation (τ_{c2} = 0.39 ns, f_2 = 0.44) probably corresponds to the local motions of the tryptophan side chains. Since the slow rotation is the prevailing component, it results in the high value of polarization experimentally observed. The value of steady-state polarization of 0.208 is consistent with the obtained correlation times and the two lifetime exponential components. Addition of guanidine drastically increased the local motion of the tryptophan residues and abolished any contribution from motion of the whole protein (Table 2). This is consistent with the expectation that under denaturing conditions, the tryptophan residues should be substantially free to rotate. Similarly, pressure denaturation resulted in an increase in local motion: the fraction of fast rotation increased from 0.44 to 0.72 and the value of the correlation time decreased from 0.39 to 0.1 ns (Table 2). However, a fraction of the tryptophan is still less mobile, as can be deduced from the persistent fraction of long rotations. It is noteworthy that the value of the rotation correlation time decreased to about 50% of the value of folded state.

Pressure dissociation of empty shells

The data described above suggest that the coat protein monomer is highly sensitive to pressure. To determine the effect of lattice contacts on subunit stability, we examined the effect of increasing pressure on shells of coat protein. Empty shells of coat protein can be prepared from procapsids by extraction of the scaffolding protein with 0.5 M GuHCl. These shells are topologically closed and are composed solely of coat protein. Empty procapsid shells at a concentration of 0.2 mg/ml were subjected to pressure treatment and monitored by fluorescence and light scattering.

The light scattering was initially 1.6 million counts at atmospheric pressure. As the pressure was increased to 2.25 kbar, the scattering progressively dropped reaching a final value of 1.2 million counts. The center of spectral mass of the fluorescence decreased in concert with the decrease in scattering, from a initial value of 29,226 to 29,066. This gradual drop in scattering is less than what would be expected for dissociation of the shells into monomeric subunits. Most of this drop is due to optical artifact due to window scrambling, and therefore it appears that empty coat protein shells are highly resistant to pressure dissociation.

At a constant pressure of 2.25 kbar, the temperature was lowered from 20°C to ⁻13°C (Fig. 8). This resulted in a pronounced decrease in the light scattering to a value of approximately 7000 counts, suggesting that the shells had fully dissociated into monomers. Fluorescence spectra recorded at −13°C and 2.25 kbar displayed a red shift in the tryptophan fluorescence consistent with subunit denaturation (Fig. 9). These results suggest that the procapsid shell is dissociated by decreasing temperature, an observation consistent with the hypothesis that the shells are stabilized by interactions among nonpolar residues that are usually termed "hydrophobic" interactions.

As the temperature was raised from −13°C to 20°C, maintaining the pressure at 2.25 kbar, the light scattering remained at a value of 7000 counts, indicating that there was no association of the subunits. The fluorescence spectra remained red shifted even when the temperature eventually reached 20°C, demonstrating that the protein was unable to refold under pressure (Fig. 9). Upon release of pressure the spectra underwent a blue shift, resulting in spectra that looked similar to the native forms of dissociated coat protein monomers (Fig. 9).

Gel filtration of pressure-treated samples of coat protein and shells

Size exclusion chromatography confirmed that the dissociation of the shells by low temperature and pressure led to the monomers of coat protein. Fig. 10 shows the elution pattern from a high molecular weight exclusion column (GPC500) of the sample of shells that was treated by pressure (2.25 kbar) under low temperature (−14°C) and then returned to 20°C and 1 bar. The control shells elutes in a position close to the void volume of the column (Fig. 1). Most of the treated protein eluted close to the total volume of the column, with an elution position consistent with that expected for monomeric coat protein. The elution pattern of the pressure treated procapsid shells was similar to that of the isolated coat protein monomers when analyzed using the TSK3000 column (not shown).

DISCUSSION

In this work we demonstrate the occurrence of a significant structural change in the monomeric coat protein subunit of bacteriophage P22 at pressures as low as 0.5 kbar. The nature of these changes—a red shift of the tryptophan emission, a decrease in the polarization, and increased local motion of tryptophan residues—suggest that the molecule is becoming

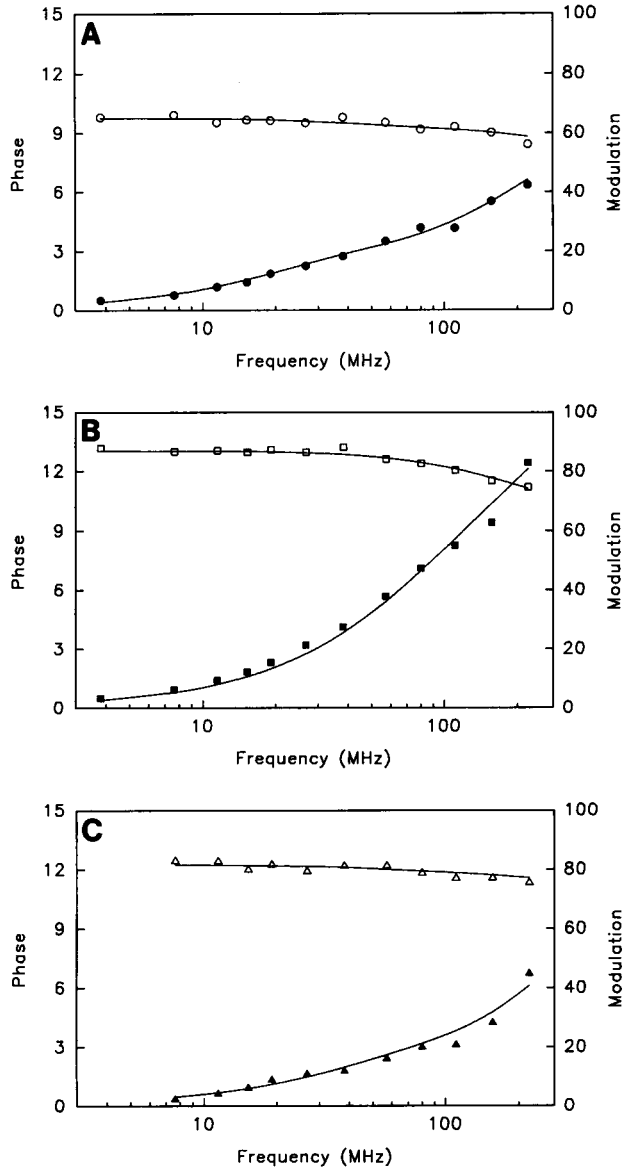

FIGURE 7 Dynamic depolarization of tryptophans in P22 coat protein. (*A*) Atmospheric pressure; (*B*) guanidine-denatured; (*C*) 2.2 kbar. Differential polarized phase angles (○) and modulation ratios (●) were measured. Curves represent the least-squares fits to the data. Excitation was at 295 nm and emission was observed through a WG 320 filter. Protein concentration was 0.2 mg/ml.

partially unfolded. This work is the third description of the denaturation of a monomeric protein at pressures below 3 kbar. Eftink et al. (1991) and Royer et al. (1993) reported pressure denaturation of staphylococcal nuclease and of a series of mutants at pressures below 3 kbar. Most single-polypeptide proteins are much less sensitive to pressure because of either smaller volume changes or larger free energies of denaturation (Silva and Weber, 1993). Despite these exceptions, in general for monomeric globular proteins, the pressure at which they denature is typically in the range of 5–12 kbar (for reviews see Heremans, 1982; Weber and Drickamer, 1983; Silva and Weber, 1993).

TABLE 2 Rotation correlation times (τ_c) for capsid protein

Capsid protein*	τ_c1	f1	τ_c2	f2	χ^2
ATM	25	0.56	0.39	0.44	2.6
GuHCl	1.1	0.37	0.21	0.63	2.4
Pressure	12	0.28	0.12	0.72	3.5

* ATM, native; GuHCl, 5.5 M guanidine-denatured; Pressure, pressure-denatured.

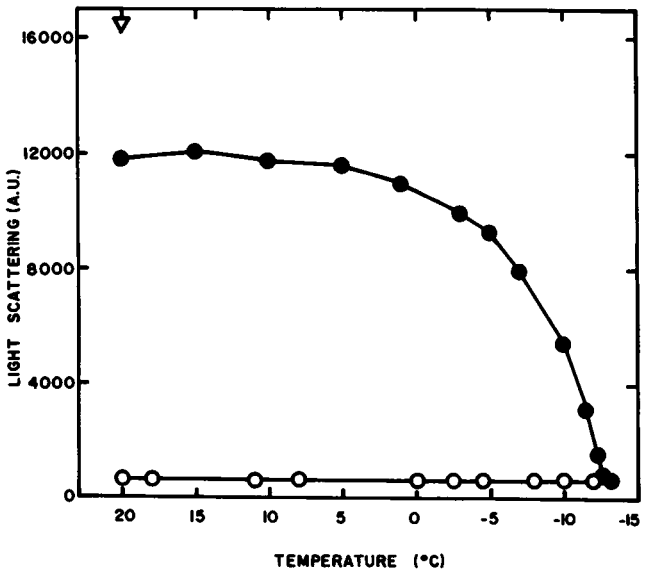

FIGURE 8 Dissociation of procapsid shell with decreasing temperature. The light scattering at 320 nm from procapsid shells at 0.2 mg/ml was recorded (*arrow, top left*), and then the pressure was increased to 2.25 kbar at 20°C. The temperature was lowered as the scattering was measured (●) and then returned to 20°C (○).

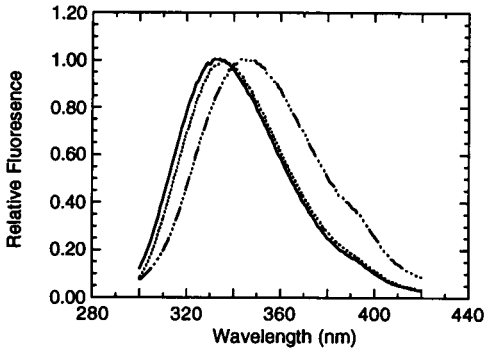

FIGURE 9 Tryptophan fluorescence emission of P22 procapsid shell (0.2 mg/ml) at atmospheric pressure (———), at 2.25 kbar, 20°C (·····), and at 2.25 kbar, −13°C (– · · · –).

Comparison of the properties of the pressure denatured monomer with those of the GuHCl-denatured monomer suggests that the conformations of the two denatured forms are not the same. The fluorescence lifetime data and measurements of tryptophan rotation suggest that in GuHCl virtually all structure is abolished, while under pressure residual struc-

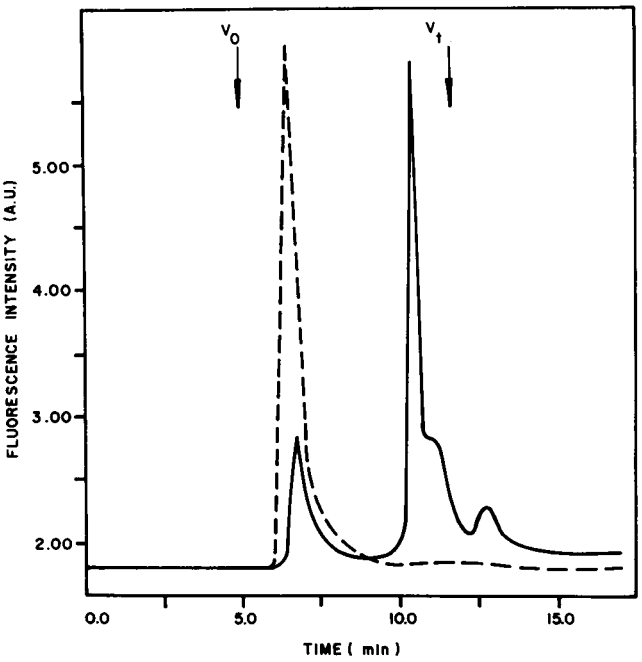

FIGURE 10 Analysis of pressure- and temperature- dissociated procapsid shells by HPLC. Procapsid shells from the experiment described in Fig. 8 were applied to a GPC500 HPLC column and analyzed at 20°C. The excluded volume (V_o) and void volume (V_t) are marked by arrows. The dashed line shows the control incubated at atmospheric pressure.

ture remains. In this respect, P22 coat protein behaves similar to Arc repressor (Silva et al., 1992; Peng and Silva, 1993) and staphylococcal nuclease (Royer et al., 1993), in which the pressure-denatured form is different from the temperature- and chemical-denatured forms.

When the monomeric subunits are incorporated into the procapsid lattice, they become very resistant to pressure denaturation. Once polymerized, they do not dissociate or denature even at pressures as high as 2.3 kbar. This increase in the stability of the coat protein monomer upon polymerization suggests that much of the stabilization energy that keeps the protein in a folded conformation is derived from interlattice contacts. This is in accord with the detailed calorimetric studies on bacteriophage T4 (Steven et al., 1992) and P22 (Galisteo and King, 1993; M. Galisteo, personal communication). For both those systems, the purified coat protein subunits shows only marginal temperature stability; however, when polymerized, the stability is greatly increased. In this respect the procapsid shells behave similarly to R17 phage particles which also cannot be dissociated by pressures of 2.5 kbar at room temperature (Da Poian et al., 1993). The R17 capsid protein dimer can be completely dissociated by pressures below 2.0 kbar. In all viral capsids with $T > 1$, the subtriangulated icosahedron requires that the protein subunits adopt position dependent conformations. The intrinsic instability of the monomer may allow the subunit to display the flexibility necessary for assembly. In view of the efforts being made toward the development of subunit-based vaccines, it will be of interest to determine how closely the stability and conformation of monomeric viral coat proteins parallels that in the assembled virion.

The procapsid shells dissociated as the temperature was decreased. They dissociated in a sharp transition which was centered at approximately −10°C. The result demonstrates the importance of entropic contributions to stabilizing the procapsid lattice. It has been recently found that some aggregates only dissociate by pressure at subzero temperatures (Foguel et al., 1992; Foguel and Weber, unpublished observations). In several cases of monomer-oligomer equilibria, the cause of association is the increase in entropy (Weber, 1993; Silva and Weber, 1993). It has previously been suggested that the primary force for formation of the procapsid was entropic in nature (Fuller and King, 1981), but as those experiments were based on in vitro procapsid assembly it was difficult to exclude the possibility that the kinetics of assembly were substantially reduced at low temperature. The higher entropy of the protein in the shells may be a result of the monomers containing solvent-exposed nonpolar side chains which become sequestered from the solvent during the formation of the procapsid shell. The native state of the monomer itself was less stable at lower temperatures (Figs. 3 and 4), indicating that the equilibrium between pressure-denatured and native states of the monomeric coat protein is also entropy-driven. It is likely that the pressure-induced denaturation of the monomer is also the result of exposure of a substantial amount of nonpolar residues to the solvent.

There are two possible means by which the dissociation could occur: one is actual cold denaturation of the protein subunit within the lattice resulting in disruption of the lattice, and the second is disruption of the lattice, followed by denaturation of the subunit. If the monomeric subunits and the assembled lattice existed in an equilibrium reaction, examining the concentration dependence of the temperature dissociation would distinguish between these possibilities. However, in the absence of scaffolding protein, the coat protein monomers will not assemble into procapsids. Direct experimental evidence for this is seen by the fact that when procapsid shells are taken to high pressure and low temperature, they dissociate. When the sample is returned to atmospheric pressure and 20°C, they do not form procapsids. If indeed the role of the scaffolding protein is to switch the coat protein subunit into a conformation active for assembly as has been suggested, pressure treatment of the subunit results in resetting of the switch.

There are two transitions in denaturation of the monomer as followed by the shift in the center of spectral mass, one occurring at low pressure and one occurring at high pressure. Similar results have been obtained for renaturation of the coat protein monomer from GuHCl (Teschke and King, 1993). The first transition becomes more cooperative and accounts for a greater degree of the total fluorescence change at low temperature. The increased cooperativity of the pressure-induced transition suggests that the protein is not only destabilized at low temperature, but also that the ensemble of molecules are more energetically homogenous. The changes

in conformation resulting from this energetic homogeneity are subtle since there are no appreciable differences in fluorescence or CD (data not shown) at atmospheric pressure over this temperature range.

Through the use of hydrostatic pressure, we can derive the denaturation equilibrium constant and the volume change of upon denaturation. The effects of pressure on the interaction between two distinct protein subunits or between intramolecular protein segments are expected to be of the same nature. The Gibbs free energy and the equilibrium constant for either reaction will depend on the volume change according to

$$K_d(p) = K_o \exp(p\Delta V/RT). \quad (3)$$

If we introduce the degree of extent of reaction at pressure $p(\alpha p)$, we can deduce the following general equation for a dissociation or a denaturation process:

$$\ln(\alpha_p^n/(1 - \alpha_p)) = p(\Delta V/RT) + \ln K_o/n^n C^{(n-1)}, \quad (4)$$

where C is the total protein concentration and K_o is the dissociation or denaturation constant. In the case of a denaturation process, the order of reaction (n) is equal to 1, and, therefore, the equilibrium does not depend on protein concentration:

$$\ln(\alpha_p/(1 - \alpha_p)) = p(\Delta V/RT) + \ln(K_o), \quad (5)$$

where

$$Kp = \alpha/(1 - \alpha),$$

where α is the degree of denaturation.

Eq. 5 permits the calculation of the standard volume change (ΔV) and the denaturation constant (K_o) at atmospheric pressure. For the fluorescence data, each transition provided a set of parameters. The sharper transition in the low pressure range has a larger volume change (100 ml/mol) than the second transition ($\Delta V = 40$ ml/mol). However, the equilibrium denaturation constants (K_o) are within the same order of magnitude: 0.05 for the first transition and 0.1 for the second. Both constants are relatively small reinforcing the idea that the coat protein is marginally stable. The volume change of the first transition is characteristic of the dissociation of globular subunits. Since the coat protein is monomeric and we did not find concentration dependence, the more likely explanation is that this phase consists of domain denaturation. The large volume change points to an interaction that should be very similar to the one that stabilizes subunits in an oligomeric protein. The transition at higher pressures (with smaller volume change) is more likely a global melting of the structure.

The lifetime studies on the native coat protein monomer suggest that there are remarkably few different environments of the tryptophan residues. Such homogeneity suggests that they may be clustered in the protein. As the protein denatures with increasing pressure, the tryptophan residues seem to exist in a larger number of environments. One possible explanation for this effect is that the structure is unfolded to the extent that the clustering is perturbed, but not to the point where all the tryptophans see a uniform environment. This interpretation fits nicely with the observation that even at 2.2 kbar, it seems that there is a significant fraction of domain rotation as measured by dynamic depolarization. Fig. 11 shows a sketch of the denaturation promoted by guanidine or by pressure. The pressure-denatured form may consist of two compacted partly folded globules connected by a polypeptide link.

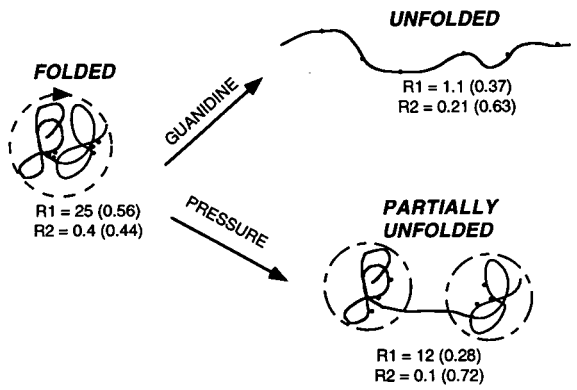

FIGURE 11 Model for the pressure and GuHCl-induced dissociation of coat protein. The folded monomer denatures completely in the presence of GuHCl, whereas under pressure the subunit partially unfolds resulting in the partial exposure of the tryptophan residues. The clustering of the tryptophan residues accounts for their relatively homogeneous lifetimes.

Why then does the protein open up with increasing pressure without completely unfolding as it does in GuHCl. Primarily, the interactions stabilizing elements of secondary structure (helices and β-sheet), are hydrogen bonds which are relatively insensitive to pressure. This is due to the fact that hydrogen bonds are permanent dipoles, and the replacement of protein-protein hydrogen bonds by protein-water hydrogen bonds is accompanied by rather small volume change. On the other hand, the replacement of weak bonds in the protein-protein interactions (London dispersion forces) by protein-water interactions is accompanied by a large volume change. Therefore, the elements of tertiary structure which are stabilized primarily by Van der Waals interactions, electrostatic interactions and relatively less by hydrogen bonds, are more pressure sensitive than those of secondary structure, and the tertiary structure is more easily perturbed than the secondary structure. This suggests that the use of pressure may be a way to populate partially unfolded intermediates under neutral solvent conditions. Indeed there are two recent reports wherein pressure has been used to populate a partially unfolded conformation of a protein (Silva et al., 1992; Peng et al., 1993).

We thank Professor Gregorio Weber for helpful suggestions and discussion and for his constant support and encouragement. We are indebted to the Laboratory for Fluorescence Dynamics at the University of Illinois for use of the lifetime facility.

This work was supported by grants from Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq), Financiadora de Estudos e Projetos (FINEP) of Brazil, and by a Grant from the European Economic

Community (CII*-CT90–0857) to J.L.S. and from United States NIH grants GM47980 (P.E.P.) and GM17890 (J.K.).

REFERENCES

Alcala, R., E. Gratton, and D. M. Jameson. 1985. A multifrequency phase fluorometer using the harmonic content of a mode-locked laser. *Anal. Inst.* 14:225–250.

Alcala, R., Gratton, E., and Prendergast, F. G. 1987a. Resolvability of fluorescence lifetime distributions using phase fluorometry. *Biophys. J.* 51: 587–596.

Alcala, R., Gratton, E., and Prendergast, F. G. 1987b. Fluorescence lifetime distributions in proteins. *Biophys. J.* 51:597–604.

Bazinet, C., J. Benbaset, J. King, J. Carazo, and J. Carrascosa. 1988. Purification and organization of the gene 1 portal protein required for phage P22 DNA packaging. *Biochemistry.* 27:1849–1856.

Beechem, J. M., and L. Brand. 1985. Time-resolved fluorescence of proteins. *In* Annual Reviews of Biochemistry. Vol. 54. C. C. Richardson, J. N. Abelson, P. D. Boyer, and A. Meister, editors. Annual Reviews, Palo Alto, CA. 43–71.

Beechem, J. M., E. Gratton, M. Ameloot, J. R. Knutson, and L. Brand. 1991. The global analysis of fluorescence intensity and anisotropy decay data: second-generation theory and programs. *In* Topics in Fluorescence Spectroscopy. Vol.2: Principles. J. R. Lakowicz, editor. Plenum Press, New York. 241–305.

Caspar, D. L. D., and A. Klug. 1962. Physical principles in the construction of regular viruses. *Cold Spring Harbor Symp. Quant. Biol.* 27:1-24.

Da Poian, A., A. Oliveira, L. P. Gaspar, J. L. Silva, and G. Weber. 1993. Pressure dissociation of RNA bacteriophage: the individuality of virus particles. *J. Mol. Biol.* 231:999–1008.

Eftink, M. R., C. A. Ghiron, R. A. Kautz, and R. O. Fox. 1991. Fluorescence and conformational stability studies of *Staphylococcus* nuclease and its mutants, including the less stable nuclease-concanavalin A hybrids. *Biochemistry.* 30:1193–1199.

Eppler, K., E. Wyckoff, J. Goates, R. Parr, and S. Casjens. 1991. Nucleotide sequence of the bacteriophage P22 genes required for DNA packaging. *Virology.* 183:519–538.

Foguel, D., R. M. Chaloub, J. L. Silva, A. R. Crofts, and G. Weber. 1992. Pressure and low temperature effects on the fluorescence emission spectra and lifetime of the photosynthetic components of Cyanobacteria. *Biophys. J.* 63:613–1622.

Foguel, D., and G. Weber. 1993. Pressure-induced dissociation, and denaturation of allophycocyanin at subzero temperatures. Submitted for publication.

Fuller, M. T., and J. King. 1981. Purification of the coat, and scaffolding protein from procapsids of bacteriophage P22. *Virology.* 112:529–547.

Galisteo, M. L., and J. King. 1993. Conformational transformations in the protein lattice of phage P22 procapsids. *Biophys. J.* 65:227–235.

Gratton, E., R. J. Alcala, and G. Marriott. 1986. Rotations of tryptophan residues in proteins. *Biochem. Soc. Trans.* 14:835–838.

Gratton, E., M. Linkeman, J. R. Lakowicz, B. Maliwal, H. Cherek, and G. Laczko. 1984. Resolution of mixtures of fluorophores using phase and modulation data. *Biophys. J.* 46:479–486.

Heremans, K. A. H. 1982. High pressure effects upon proteins and other biomolecules. *Annu. Rev. Biophys. Bioeng.* 11:1–21.

Jaenicke, R. 1987. Folding and association of proteins. *Prog. Biophys. Mol. Biol.* 49:117–237.

King, J., and S. Casjens. 1974. Catalytic head assembly protein in virus morphogenesis. *Nature.* 251:112–119.

Lakowicz, J. R., G. Laczko, H. Cherek, E. Gratton, and M. Linkeman. 1984. Analysis of fluorescence decay kinetics from variable-frequency phase shift and modulation data. *Biophys. J.* 46:463–477.

Paladini, A., and G. Weber 1981. Pressure-induced reversible dissociation of enolase. *Biochemistry.* 20:2587–2593.

Peng, X., J. Jonas, and J. L. Silva. 1993. Molten globule conformation of arc repressor monomers determined by high pressure (^{1}H) NMR spectroscopy. *Proc. Natl. Acad. Sci. USA.* 90:1776–1780.

Prasad, B. V. V., P. E. Prevelige, E. Marietta, R. O. Chen, D. Thomas, J. King, and W. Chiu. 1993. Three dimensional transformation of capsids associated with genome packaging in a bacterial virus. *J. Mol. Biol.* 231: 65–74.

Prevelige, P. E., Jr., and G. D. Fasman. 1987. Structural studies of acetylated and control inner core histones. *Biochemistry.* 26:2944–2955.

Prevelige, P. E., D. Thomas, and J. King. 1988. Scaffolding protien regulates the polymerization of P22 coat subunits into icosahedral shells in vitro. *J. Mol. Biol.* 202:743–757.

Prevelige, P. E., D. Thomas, and J. King 1993. Nucleation and growth phases in the polymerization of coat and scaffolding subunits into icosahedral procapsid shells. *Biophys. J.* 64:824–835.

Rossmann, M. G. 1984. Constraints on the assembly of spherical virus particles. *Virology.* 134:1–13.

Rossmann, M. G., and J. E. Johnson. 1989. Icosahedral RNA virus structure. *In* Annual Reviews of Biochemistry. Vol 58. C. C. Richardson, J. N. Abelson, P. D. Boyer, and A. Meister, editors. Annual Reviews, Palo Alto, CA. 533–573.

Royer, C. A. 1992. Investigation of the structural determinants of the intrinsic fluorescence emission of the trp repressor using single tryptophan mutants. *Biophys. J.* 63:741–750.

Royer, C. A, J. A. Gardner, J. M. Beechem, J. C. Brochom, and K. S. Matthews. 1990. Resolution of the fluorescence decay of the two tryptophan residues of lac repressor using single tryptophan mutants. *Biophys. J.* 58:363–378.

Royer, C. A., A. P. Hinck, S. N. Loh, K. E. Prehoda, X. Peng, J. Jonas, and J. L. Markley. 1993. Effects of amino acid substitutions on the pressure denaturation of staphylococcal nuclease as monitored by fluorescence and nuclear magnetic resonance spectroscopy. *Biochemistry.* 32:5222:5232.

Royer, C. A., G. Weber, T. J. Daly, and K. S. Matthews. 1986. Dissociation of the lactose repressor protein tetromer using high hydrostatic pressure. *Biochemistry.* 25:8308–8315.

Silva, J. L., E. W. Miles, and G. Weber. 1986. Pressure dissociation and conformational drift of the beta dimer of tryptophan synthase. *Biochemistry.* 25:5780–5786.

Silva, J. L., C. F. Silveira, A. Correia, and L. Pontes. 1992. Dissociation of a native dimer to a molten globule monomer. Effects of pressure and dilution on the association equilibrium of Arc repressor. *J. Mol. Biol.* 223:545–555.

Silva, J. L., M. Villas-Boas, C. F. S. Bonafe, and N. C. Meirelles. 1989. Anomalous pressure-dissociation of large protein aggregates. Lack of concentration dependence and irreversibility at extreme degrees of dissociation of extracellular hemoglobin. *J. Biol. Chem.* 264: 15863–15868.

Silva, J. L., and G. Weber. 1988. Pressure-induced dissociation of Brome mosaic virus. *J. Mol. Biol.* 199:149–161.

Silva, J. L., and G. Weber. 1993. Pressure stability of proteins. *Annu. Rev. Phys. Chem.* 44:89:113..

Steven, A. C. 1993. Conformational change—an alternative energy source? Exothermic phase transition in phage capsid maturation. *Biophys. J.* 65: 5–6.

Steven, A. C., H. L. Greenstone, F. P. Booy, L. W. Black, and P. D. Ross. 1992. Conformational changes of a viral capsid protein. Thermodynamic rationale for proteolytic regulation of bacteriophage T4 capsid expansion, co-operativity, and super-stabilization by soc binding. *J. Mol. Biol.* 228: 870–884.

Teschke C. M., and J. King. 1993. Folding of the phage P22 coat protein in vitro. *Biochemistry.* 32:10839–10847.

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

Weber G. 1993. Thermodynamics of the association, and the pressure dissociation of oligomeric proteins. *J. Phys. Chem.* 97:7108–7115.

Weber, G., and H. G. Drickamer. 1983. The effects of high pressure upon proteins and other biomolecules. *Q. Rev. Biophys.* 116:89–112.