Differences in Pressure Stability of the Three Components of Cowpea Mosaic Virus: Implications for Virus Assembly and Disassembly†

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ABSTRACT: A comparison of pressure stability of empty capsids and ribonucleoprotein particles of cowpea mosaic virus (CPMV) is presented. A combination of high pressure and subdenaturing concentrations of urea was utilized to promote dissociation and denaturation. We found that RNA plays an important role in stabilizing the particles as well as in conferring reversibility to the pressure-induced denaturation. Dissociation and denaturation of the top component (empty capsid) was observed at 2.5 kbar and in the presence of 2.5 M urea. The pressure-dissociated state of the capsid protein had the characteristics of a denatured conformation as suggested by fluorescence spectra, lifetime of tryptophans, and binding of bis-ANS. The properties of the dissociated capsid protein were more similar to those of a molten-globule conformation, different from the more drastically unfolded state obtained using high concentrations of urea. Whereas the fluorescence of bis-ANS increased for the pressure-dissociated protein (1.5 M urea and 2.5 kbar), it decreased for the virus denatured by 6.0 M urea. Middle and bottom components underwent less than 50% change in center of spectral mass at 2.5 kbar and 2.5 M urea. The particles containing RNA could be fully affected by pressures of 2.5 kbar—as measured by the spectral shift—only in the presence of 5.0 M urea. RNA-containing capsids denatured by pressure did not bind bis-ANS, suggesting that the capsid protein continues to be bound to the RNA after the protein–protein contacts are broken by pressure. Reassembly of the nucleoprotein particles was obtained after decompression, reinforcing the idea that proteins had not dissociated from RNA. In contrast, top component disassembly was irreversible: after decompression the coat proteins remain dissociated and partially denatured as shown by fluorescence, light scattering, and bis-ANS binding. RNA promotes renaturation and increases the pressure stability by 60 kcal per mol of particle, demonstrating the crucial role of energy coupling between protein association and binding to RNA for virus assembly.

Cowpea mosaic virus (CPMV) is the type member of the Comoviridae, a family of plant viruses that have a bipartite, single-stranded, positive-sense RNA genome. CPMV capsids contain equimolar amounts of a large (L) and a small (S) protein with molecular weights of 42 000 and 23 000, respectively (Wu & Bruening, 1971). The two RNA molecules are encapsidated into distinct particles. In addition, empty shells are formed *in vivo*. Thus, preparations of these viruses result in three different components that can be separated by gradient ultracentrifugation: the top (no RNA), the middle (smaller RNA), and the bottom (larger RNA) fractions. The three components have the same protein composition, although they differ in RNA content. The bottom component contains RNA 1 ($M_r = 2.04 \times 10^6$), while the middle component contains RNA 2 ($M_r = 1.22 \times 10^6$). These RNA molecules correspond to 34% and 25% of the particle weight, respectively (van Kammen, 1967). Isolated RNA 1 can replicate independently in cowpea protoplasts (Goldbach *et al.*, 1980). However, both bottom and middle components are necessary to complete the infection process (Bruening & Agrawal, 1967; van Kammen, 1968), since both coat proteins are encoded by the RNA 2 (Franssen *et al.*, 1982).

Three-dimensional electron microscopy image reconstruction has shown that the CPMV particles present an icosahedral symmetry (Crowther *et al.*, 1974). Large crystals of the CPMV particles were obtained for the first time by White and Johnson (1980). A preliminary analysis at 3.5-Å resolution has shown that the CPMV structure is very similar to that of animal picornaviruses (Stauffacher *et al.*, 1987). One coat-protein heterodimer forms each of the 60 asymmetric units of a $P = 3$ capsid (Rossmann & Johnson, 1989). The purified middle component of bean pod mottle virus, another member of the comovirus family, was also crystallized (Sehnke *et al.*, 1988). Its X-ray diffraction map at 3.0-Å resolution was used to construct a complete three-dimensional model of the capsid (Chen *et al.*, 1989). In this study it was possible to detect well ordered RNA which interacts with the 60 asymmetric units of the particle. An analysis of these data made possible the construction of a model for the RNA packing in BPMV middle component (Chen *et al.*, 1990).

Although much progress has been made on the understanding of viral structure in the last years, the thermodynamics and the mechanism of virus assembly are still poorly understood. Hydrostatic pressure is an efficient tool for promoting dissociation of oligomeric proteins (Weber & Drickamer, 1983; Silva & Weber, 1993) as well as viral structures (Silva & Weber, 1988; Silva *et al.*, 1992a; Silva, 1993). Pressure dissociation studies may also help to clarify the role of protein–nucleic acid interactions in virus assembly

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and stability. Hydrostatic pressure has been successfully utilized to analyze the stabilization of subunit interactions conferred by the nucleic acid in the Arc repressor–operator DNA complex (Silva & Silveira, 1993). In addition, studies with the R17 bacteriophage have shown that the coat-protein dimer is less stable than the whole phage (Da Poian *et al.*, 1993). The pressure effects on proteins can be followed by optical methods such as light scattering (Engelborghs *et al.*, 1976; Silva *et al.*, 1989), fluorescence emission, and polarization (Paladini & Weber, 1981; Silva *et al.*, 1986).

In this work we describe the pressure denaturation of CPMV components. We employ hydrostatic pressure and urea to promote capsid denaturation. We use the general term "pressure denaturation" to refer to the spectral shift of tryptophan emission. This, however, does not mean that there is complete unfolding. We find that the presence of RNA strongly stabilizes the capsid against denaturation. We also show that while pressure denaturation of the bottom and middle components is completely reversible, the top component cannot reassemble after dissociation.

MATERIALS AND METHODS

(*a*) *Chemicals.* All reagents were of analytical grade. Distilled water was filtered and deionized through a Millipore water purification system. The experiments were performed at 20 °C in the standard buffer: 50 mM Tris, 150 mM NaCl, pH 7.5. Tris was chosen as the buffer because the dependence of its pK_a on pressure is small. At 3.0 kbar, the value of pK_a increases by only 0.1 (Neuman *et al.*, 1973).

(*b*) *CPMV Purification.* The Bil mutant of the yellow strain of cowpea mosaic virus was propagated in blackeyed peas. The initial innoculum was kindly supplied by Professor George Bruening (University of California, Davis). The virus was purified as described elsewhere (Siler *et al.*, 1976; White & Johnson, 1980). The three components were separated by equilibrium centrifugation in a self-forming 40% cesium chloride gradient at pH 7.0 (Schmidt *et al.*, 1983).

(*c*) *Spectroscopic Measurements under Pressure.* The high-pressure bomb has been described by Paladini and Weber (1981) and was purchased from SLM-AMINCO (Urbana, IL). Fluorescence spectra and light-scattering measurements were recorded on ISS200 or on ISSK2 spectrofluorometers (ISS Inc., Champaign, IL). Light scattering at 320 nm was measured at 90° in the spectrofluorometer by selecting the same wavelength for both excitation and emission monochromators. Fluorescence spectra at pressure p were quantitated by the center of spectral mass $\langle \nu_p \rangle$.

$$\langle \nu_p \rangle = \sum \nu_i F_i / \sum F_i \qquad (1)$$

where 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 pressure was increased by steps of 200 bar. The sample was allowed to equilibrate for 10 min prior to making measurements. There were no time-dependent changes in either fluorescence spectra or light scattering between 5 and 60 min.

The degree of dissociation (α_p) is related to $\langle \nu_p \rangle$ by the expression

$$\alpha_p = [1 + Q(\langle \nu_p \rangle - \langle \nu_f \rangle)/(\langle \nu_i \rangle - \langle \nu_p \rangle)]^{-1} \qquad (2)$$

where 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 corresponding quantities for dissociated and associated forms.

Fluorescence polarization measurements were made in a ISSK2 fluorometer (ISS Inc., Champaign, IL) using the L-format mode. The corrections for the scrambling of the windows were performed as described by Paladini and Weber (1981).

(*d*) *Tryptophan Lifetime Measurements.* Lifetime measurements were performed by a multifrequency cross-correlation phase and modulation fluorometer that 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; Silva *et al.*, 1992b). Techniques used for phase fluorometry lifetime measurements and data analysis have been fully described previously (Lakowicz *et al.*, 1984; Gratton *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 with a cutoff at 320 nm. Measurements under pressure were performed as previously described (Silva *et al.*, 1992b; Foguel *et al.*, 1992). 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.

(*e*) *Size Exclusion Chromatography.* High-performance liquid chromatography was carried out in a prepacked SynChropak GPC500 column (250 × 4.6 mm inner diameter), obtained from SynChropak Inc. (Linden, IN). The system was equilibrated in 50 mM Tris–0.2 M sodium acetate buffer (pH 7.0). A flow rate of 0.3 mL/min was utilized. Sample elution was monitored by fluorescence at 330 nm (excitation at 280 nm) and absorbance at 260 or 280 nm.

RESULTS

(*a*) *Effects of Pressure on Top Component.* The three components of CPMV do not denature at pressures up to 2.5 kbar: The values of the center of spectral mass and light scattering did not change significantly for the three components at 2.5 kbar when compared to measurements for the native virus. On the other hand, these values were strongly modified for urea-denatured virus. This behavior is similar to that found for other viruses such as R17 bacteriophage (Da Poian *et al.*, 1993). As the dissociation of R17 is facilitated by a combination of pressure with subdenaturing concentrations of urea at atmospheric pressure, the effects of urea on CPMV were also investigated (Figure 1). The tryptophan fluorescence spectra of top component (empty capsids) changed significantly only at urea concentrations higher than 3.0 M (Figure 1A). The elution profile of top component in a size exclusion HPLC column was not affected by 2.5 M urea (Figure 2A,B). Therefore, we have utilized urea concentrations lower than 2.5 M in investigating the pressure dissociation of empty capsids.

Figure 1B shows the changes in the center of spectral mass and in light scattering of the top component in the absence and in the presence of 2.5 M urea. In the latter condition, a large red shift and a large decrease in the light scattering were observed as pressure was applied, suggesting that the virus particle dissociated at 2.5 kbar. The decrease in the light scattering promoted by pressure is compatible with a decrease in size of more than 10-fold. Because of the low

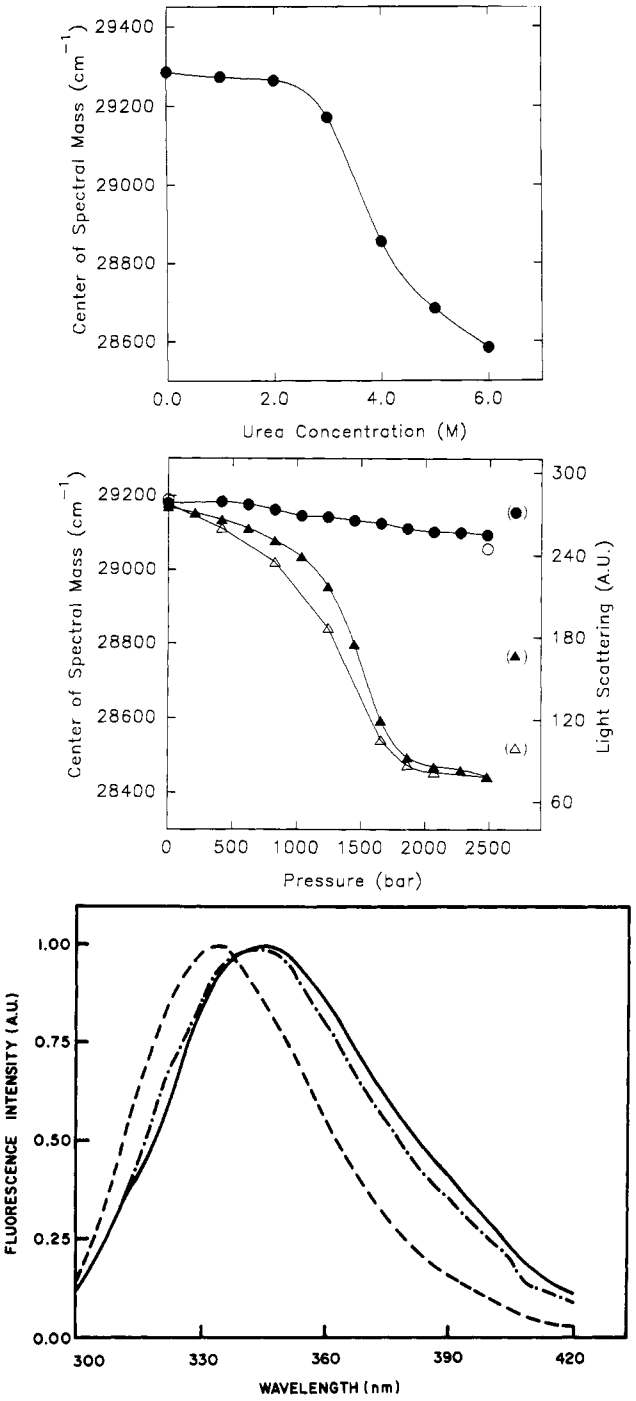

FIGURE 1: Spectra and light scattering of CPMV top component. (A, top) Effect of urea on the center of spectral mass of top component. (B, middle) Effect of pressure on the fluorescence emission (●, ▲) and on the light scattering (O, △) of the top CPMV, in the absence (●, O) and in the presence (▲, △) of 2.5 M urea. The symbols in parentheses correspond to the respective values after pressure release. (C, bottom) Tryptophan fluorescence spectra of top component in the presence of 2.5 M urea at atmospheric pressure (– – –), at 2.5 kbar (—), and in the presence of 6.0 M urea at atmospheric pressure (–•–). The virus concentration was 50 μg/mL. For the fluorescence experiment, the samples were excited at 280 nm and the emission was measured from 300 to 420 nm. For the light scattering, excitation was at 320 nm and scattering was measured at 90° at 320 nm.

value of light scattering at the endpoint, which is superimposed on a background signal due to reflection, we cannot determine precisely the size of the dissociated protein. We will consider the general term pressure denaturation to refer to the spectral shift of tryptophan emission. Evidence for a combination of dissociation and denaturation is provided by the superposition

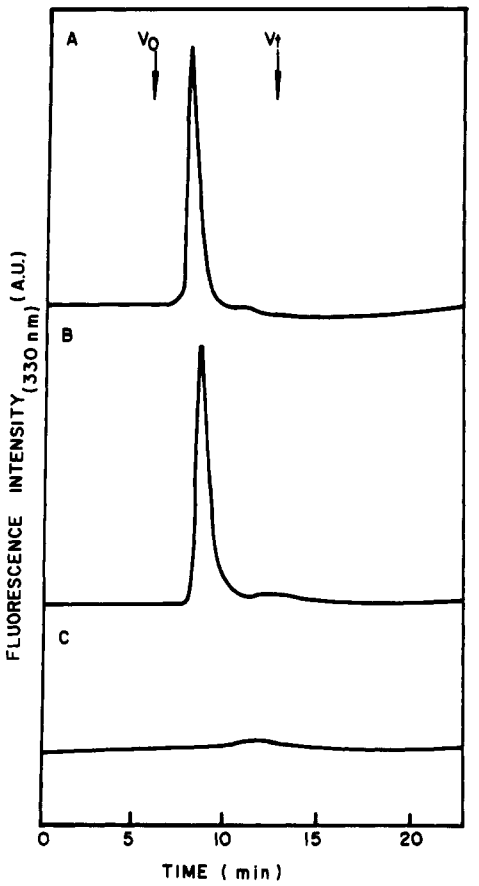

FIGURE 2: HPLC of native and pressure-denatured CPMV top component. Size-exclusion high-performance liquid chromatography of (A) native particles of top component, (B) top component in presence of 2.5 M urea, and (C) top component subjected to 2.5 kbar in the presence of 2.5 M urea. Elution of the protein was monitored by intrinsic fluorescence (λ_{exc} = 280 nm; λ_{em} = 330 nm).

of the spectrum obtained at 2.5 kbar in the presence of 2.5 M urea and that obtained for virus particles dissociated and denatured by 6.0 M urea (Figure 1C). The top component dissociation was not reversible, based on the observation that the light scattering did not increase significantly after decompression (Figure 1B). On the other hand, there was about 45% recovery of the center of spectral mass, which could be related to a conformational change or to a dimerization (or any limited oligomerization) of the virus subunits.

Size-exclusion chromatography confirmed that the top component dissociation was not reversible (Figure 2). The peak that corresponds to the virus elution at atmospheric pressure (Figure 2A,B) disappeared in the pressurized sample (Figure 2C). This result was also obtained for the urea-denatured virus (not shown) and might be related to the adsorption of the dissociated proteins on the column. It should be pointed out that the protein was in solution before injection in the column and that its light-scattering and fluorescence spectra were characteristic of dissociated subunits (Figure 1).

Pressure-induced denaturation was facilitated by increasing urea concentration (Figure 3A). The $p_{1/2}$, the pressure necessary to promote half denaturation, shifted to lower pressures as more urea was added to the sample. From pressure-dissociation data, it is possible to calculate both the standard volume change of association (ΔV) and the dissociation constant at atmospheric pressure (K_{do}), based on the thermodynamic relation:

$$K_d(p) = K_{do} \exp(p\Delta V/RT) \quad (3)$$

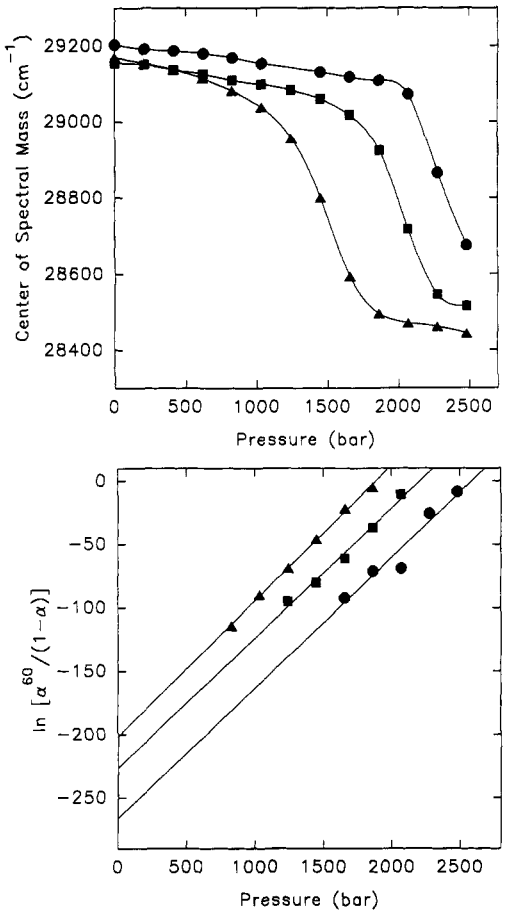

FIGURE 3: Effect of urea on CPMV top component denaturation by pressure. (A, top) Plot of center of spectral mass as a function of pressure for top CPMV in the presence of 0.75 (●), 1.5 (■), and 2.25 M urea (▲). (B, bottom) Plot of ln $[\alpha_p^{60}/(1-\alpha_p)]$ versus pressure in the presence of 0.75 (●), 1.5 (■), and 2.25 M urea (▲). The virus concentration was 200 μg/mL.

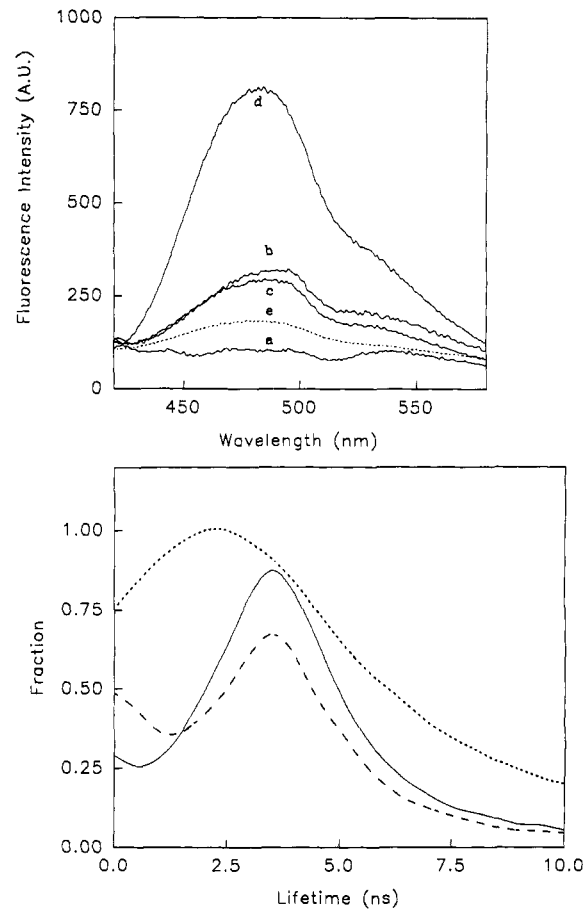

FIGURE 4: (A, top) Binding of bis-ANS to native and pressure-denatured particles. Bis-ANS in water (a). The probe was incubated with top CPMV at atmospheric pressure in the presence of 1.5 M urea (b) and in the presence of 6.0 M urea (c), and at 2.5 kbar in the presence of 1.5 M urea (d). The dashed line corresponds to the binding of bis-ANS to bottom component particles at 2.5 kbar in the presence of 5.0 M urea (e). Bis-ANS final concentration was 2 μM. The excitation wavelength was 360 nm, and the emission was measured from 420 to 580 nm. (B, bottom) Lifetime distributions of top component tryptophans. Phase and modulation in the frequency range of 5–300 MHz were measured as described under Materials and Methods. The excitation wavelength was 295 nm, and the emission was observed through a WG 320 filter. The protein concentration was 100 μg/mL. Lifetime analysis was performed using two Lorentzian distributions: top component in the presence of 1.5 M urea at atmospheric pressure (—) and 1.5 M urea + 2.5 kbar (– – –) and in the presence of 6.0 M urea at atmospheric pressure (- - -).

where $K_d(p)$ is the dissociation constant at pressure p. It is acceptable to treat ΔV as a pressure-independent constant due to the low compressibility of proteins (Gavish *et al.*, 1983; Gekko & Hasegawa, 1986). If we introduce the degree of dissociation at pressure p, α_p, we can write eq 3 for the case of a dissociation reaction between a protein aggregate and its n dissociated subunits:

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

Equation 4 permits the calculation of the standard volume change (ΔV) from measurements at a fixed protein concentration, at a series of different pressures. We found that the volume change of association did not vary with the increase in urea concentration (Figure 3B). Assuming that the whole viruses dissociate into dimers ($n = 60$), the ΔV value obtained was 2000 mL/mol of capsid. The value of $\Delta V/n$ (33 mL/mol) did not vary significantly for n values larger than 5 and is more appropriate in our case since we do not know the final oligomeric size of the dissociated capsid protein.

(*b*) *Binding of bis-ANS and Tryptophan Fluorescence Lifetime of Top Component.* The fluorophore bis-ANS [bis-(8-anilinonaphthalene-1-sulfonate)] binds noncovalently to apolar segments in proteins, especially in proximity to positive charges (Rosen & Weber, 1969). Its binding is accompanied by a large increase in its fluorescence quantum yield and has been used to probe protein structural changes (Horowitz *et al.*, 1984; Silva *et al.*, 1992b). Figure 4A shows that top component, in the presence of 1.5 M urea at atmospheric pressure, bound a small amount of bis-ANS. Exactly the same spectrum was obtained for the native virus in the absence of urea (not shown). The top component denatured by 6.0 M urea bound slightly less bis-ANS than the native particle (absence of urea). On the other hand, top component dissociated and denatured by pressure in subdenaturing concentrations of urea (1.5 M) presented a 5-fold increase in the emission of the probe (Figure 4A). The increased binding of anilinonaphthalenesulfonate probes is strong evidence that the protein assumes a molten-globule conformation (Goto & Fink, 1989). The molten globules are compact, partially folded states that partially expose the hydrophobic core to the aqueous medium (Ptitsyn, 1987; Creighton, 1988; Matouschek *et al.*, 1990). Therefore, whereas pressure promotes the denaturation of empty CPMV particles to a molten-globule state, high concentrations of urea lead to a more disorganized structure (completely unfolded state), where less bis-ANS is bound.

The excited-state lifetime of tryptophanyl residues present in top component was measured for the native virus and for

Table 1: Results of Continuous Distribution and Discrete Exponential Analysis for the Lifetime Data

condition	1.5 M urea–atm	1.5 M urea–2.5 kbar	6.0 M urea–atm
	Continuous Distributions		
center 1 (ns)	3.464	3.390	2.201
width 1 (ns)	3.356	3.469	7.765
fraction 1	0.877	0.695	1.000
center 2 (ns)	0.000	0.002	
width 2 (ns)	0.824	1.800	
fraction 2	0.123	0.305	
χ^2	4.24	5.82	5.25
	Two Discrete Exponentials		
center 1 (ns)	4.366	4.327	6.128
fraction 1	0.776	0.696	0.732
center 2 (ns)	0.519	0.526	0.804
fraction 2	0.224	0.304	0.268
χ^2	49.7	57.6	112

both pressure and urea-denatured coat proteins. Figure 4B shows the best fits obtained using a two-component Lorentzian distribution for the phase and modulation data for top component in the presence of 1.5 M urea, at atmospheric pressure, and for top proteins denatured by 2.5 kbar in the presence of 1.5 M urea and denatured by 6.0 M urea at atmospheric pressure. The fits were obtained using a lifetime distribution approach (Alcala *et al.*, 1987a,b) rather than a sum of exponentials. The χ^2 values for each fit as well as the values of the center, width, and fraction of the distributions are summarized in Table 1. This table also shows the poor fit (more than 10-fold higher χ^2) that is obtained using a decay model based on two discrete exponentials. The denaturation by urea promoted a decrease in the value of the center of the distribution with a great increase in the half-width, and the components merged into a single distribution. The increase in the half-width suggests a more heterogeneous environment of tryptophans than in the native virus.

The denaturation by pressure promoted a more limited alteration in the distribution. There was an increase in the fraction of the first component (shorter lifetimes) and practically no change in the width of the second component. These data are similar to those found by Fernando and Royer (1992) with Trp repressor: at intermediate concentrations of urea (1.8 M), a condition in which the protein is not unfolded, the width of the distribution decreases; at higher concentrations of urea the distribution is much more heterogeneous. Our lifetime data suggest that the conformation of the pressure-denatured state is different from that of the high-urea denatured state. It is noteworthy that the spectra are in both cases completely red-shifted.

(*c*) *Role of the RNA in CPMV Stability.* In order to compare the stability of the three CPMV components, the effects of pressure on each component were checked in the presence of 2.5 M urea (Figure 5). Clearly, the top component is much less stable than the ribonucleoprotein particles. In a pressure-dissociation experiment, the magnitude of stabilization of the subunit interaction by the RNA can be determined by the expression (Silva & Silveira, 1993)

$$\Delta\Delta G = -[(p_{1/2})_R \Delta V_R/n - (p_{1/2})_o \Delta V_o/n] \quad (5)$$

where $(p_{1/2})_R$ and $(p_{1/2})_o$ are, respectively, the midpoints for pressure dissociation of the ribonucleoprotein and of the empty capsids, and $\Delta V_R/n$ and $\Delta V_o/n$ are the volume changes of association per subunit for the ribonucleoprotein and for the empty capsids, respectively. The values of $\Delta V_R/n$ and $\Delta V_o/n$ were the same for the bottom, middle and top components (33.3 mL/mol). Therefore, eq 5 can be simplified to

$$\Delta\Delta G = -[(p_{1/2})_R - (p_{1/2})_o]\delta V = -\Delta p_{1/2}\delta V \quad (6)$$

where $\delta V = \Delta V_R/n = \Delta V_o/n$.

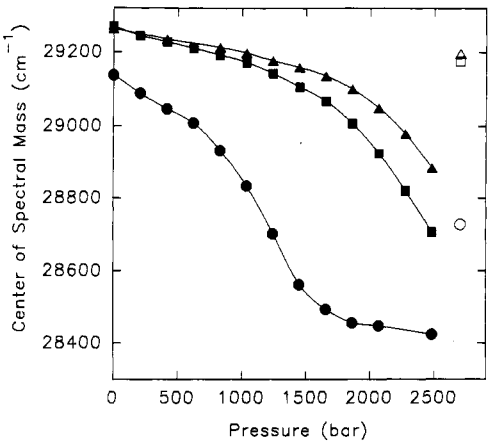

FIGURE 5: Pressure stability of the three components of CPMV. Pressure denaturation of the top (●), middle (▲), and bottom (■) components in the presence of 2.5 M urea. The hollow symbols correspond to the values after return to atmospheric pressure.

The presence of the RNA shifts the $p_{1/2}$ by 1241 bar (Figure 5), which corresponds to an increase of 988 cal/mol in the free energy per subunit. For these calculations, we used as reference the endpoint observed for denatured top component. We have found that this same endpoint is reached when middle and bottom components are fully denatured by a combination of high pressure and subzero temperatures (unpublished results).

(*d*) *Role of the RNA in the Reversibility of the Pressure Effects.* As shown before, the denaturation of the top component was completely irreversible (Figures 1 and 2). In order to examine the reversibility of pressure denaturation of the middle and bottom components, we performed the denaturation in the presence of 5.0 M urea, a condition in which both middle and bottom components could be completely denatured by pressure as determined by the center of spectral mass (Figure 6A). The most important observation in this experiment is that the RNA-containing particles could renature after pressure release (Figure 6). After decompression, the center of spectral mass returned to a value close to that of the nonpressurized control (Figure 6A). Size-exclusion chromatography confirmed the reversibility of the middle and bottom components denaturation. The pressurized samples of both bottom and middle CPMV presented an elution pattern similar to that observed for the nonpressurized samples. Figure 6B shows the elution of bottom component before pressurization and after the pressure was released. The same result was obtained for denaturation of the middle component (not shown).

Bis-ANS bound similarly to the three native components of CPMV. The fluorescence spectra of the probe incubated with top and bottom components at atmospheric pressure were exactly the same. However, bis-ANS binding during pressure denaturation changed in opposite directions for empty and ribonucleoprotein particles. While pressure denaturation led top-component proteins to bind more bis-ANS, the opposite occurred in middle and bottom components (compare spectra d and e in Figure 4A). As deduced from the tryptophan emission spectra, pressure induces partial denaturation of the

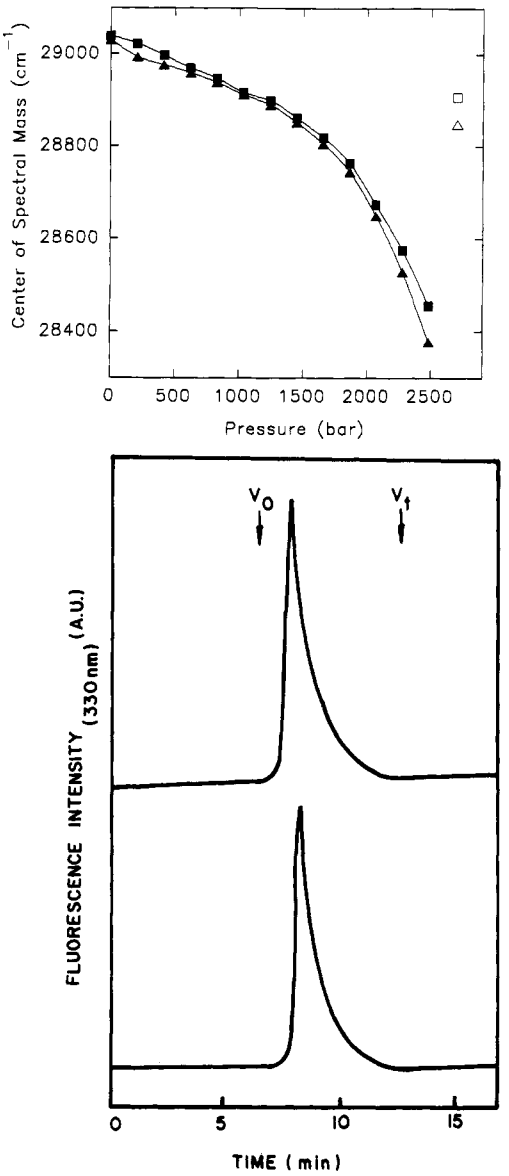

FIGURE 6: Reversibility of middle and bottom component dissociation. (A, top) Plot of the center of spectral mass versus pressure for the middle (▲) and bottom (■) CPMV components in the presence of 5.0 M urea. The hollow symbols correspond to the values after decompression to 1 bar. (B, bottom) Size exclusion high-performance liquid chromatography of the bottom component in the presence of 5.0 M urea before (top panel) and after (bottom panel) pressurization at 2.5 kbar. The elution of the protein was monitored by intrinsic fluorescence (λ_{exc} = 280 nm; λ_{em} = 330 nm).

protein components and probably disruption of the protein–protein interactions. However, the capsid proteins seem to continue to bind RNA; this results in a decreased binding of bis-ANS. Because of its negative charge, bis-ANS has a high affinity for nonpolar pockets containing few positive charges.

In order to obtain information about the mobility of the ribonucleoprotein capsid during pressure denaturation, we measured polarization of tryptophan emission. The steady-state polarization for the middle CPMV particles was 0.1450. This relatively low value suggests that Trp residues already have local or segmental motion in the capsid. Upon application of 2.5 kbar pressure, the polarization decreased to 0.0910, reflecting an increase in the dynamics of the capsid protein. Increased segmental and local rotations support the idea that the icosahedral particles are disassembled by pressure and the tryptophans in the capsid protein gain more freedom to rotate, even though the protein still binds the RNA.

DISCUSSION

We have measured the stability of three forms of the $P = 3$ icosahedral virus CPMV. We report two main findings: (1) The capsid of CPMV is stabilized against pressure and urea disruption by the presence of the viral RNA; (2) the renaturability of denatured capsids is also affected by the viral RNA—conditions of pressure and urea concentration that irreversibly denature RNA-free capsids cause only reversible effects when the RNA is present.

(*a*) *How Is a Partially Unfolded Conformation Related to Capsid Assembly?* The properties of the dissociated capsid protein were characteristic of a molten-globule conformation, different from the more drastically unfolded state obtained using high concentrations of urea. The increase in bis-ANS binding provides strong support for a molten-globule conformation. The lifetime studies on the native top component suggest that there is considerable heterogeneity in the environments of the tryptophan residues. As the particle denatures with increasing pressure, the tryptophan residues seem to keep a rather similar dispersion, although they are definitely highly exposed to water as determined from the low energy of the emission. One possible explanation for this effect is that the structure is denatured to the extent that the tryptophans become exposed to water; but they continue to see some of the native environment during the excited state because most of the secondary structure is maintained, as would be expected for a partially unfolded state (Ptitsyn, 1987; Creighton, 1988; Matouschek *et al.*, 1990; Silva *et al.*, 1992b).

On the other hand, the lifetime distribution of the capsids denatured by a high concentration of urea changes drastically, showing unequivocally that this state is distinct from the one obtained by pressure. High pressure would affect primarily the quaternary and tertiary structure of the protein, leading to a partially denatured conformation, whereas high urea concentrations would disrupt the secondary structure as well. A native-like secondary structure without specific tertiary structure is one of the properties of a molten globule (Ptitsyn, 1987; Creighton, 1988; Goto & Fink., 1989).

If one wants to make a large multiassembly complex such as that of a 120-subunit icosahedron, one needs to have a strategy for making the capsid much more stable than the dissociated subunits. We find here that indeed the dissociated capsid protein of the CPMV has a conformation that is closer to that of a partially unfolded state. There must be a switch to convert this conformation, which is a nonassociable state, to the subunits ready to associate. The pressure-denatured virus particles in the presence of RNA (middle and bottom CPMVs) do not bind bis-ANS, although we suppose that all of the protein–protein contacts are broken on the basis of the red shift of tryptophan emission and the lower polarization. This can be interpreted as a more preserved conformation, probably because the capsid is broken but continues to be bound to the RNA. This state is probably more native than that of the pressure-denatured top component; this once more suggests a key role for RNA as a chaperone during assembly of the capsid. In the absence of RNA the subunits drift to a disorganized structure and cannot renature when the perturbation is withdrawn. The capsid protein of the P22 procapsid, a ds-DNA virus, also assumes a conformation similar to a molten globule when dissociated by pressure (Prevelige *et al.*, 1994). In that case the isolated capsid protein is a monomer that is easily denatured by pressure, and reassembly only occurs if scaffolding protein is added to the medium to act as a chaperonin. Like the P22 monomers, the

dimer of R17 phage is very unstable, either to pressure or to denaturants (Da Poian *et al.*, 1993).

(*b*) *Implications of the Differences in Pressure Stability for Assembly.* Because of the direct relation between pressure and free energy ($\Delta G = \Delta p \Delta V$), the degree of stabilization can be quantified. The difference in free energy between middle and top components gives, for the first time, the contribution of the RNA to the stabilization of the capsid. The free-energy interaction per subunit in the middle component is more stable than in the top component by 1 kcal/mol, which means that a stabilization conferred by RNA on the whole virion is 60 kcal/mol. In light of the fact that the final conformational states of denatured empty and ribonucleoprotein particles are different, the calculated difference in free energies has a combined meaning (thermodynamic and kinetic); nevertheless, we can infer that the binding of RNA is coupled with a favorable interaction between the subunits to generate the virus capsid. The much lower stability of empty capsids shows the role of RNA in assembly of the virus particles and guarantees the higher yield of capsids containing RNA. The infectious particles of a virus must be very stable to have their structures preserved while they are out of the host cell environment or being transported from host to host by a vector. In the case of CPMV, the transmission vector is a beetle whose regurgitant (the natural medium for the virus) contains very high levels of ribonuclease. In spite of this, the particles retain their infectivity even during a long period within the beetle (Gergerich *et al.*, 1986; Gergerich & Scott, 1988). Our data show a clear difference between the stability of the infectious particles (middle and bottom CPMV) and the empty shells (top component). We can infer from these findings that the stability is related to the presence of the RNA in the middle and bottom particles. Energy coupling between protein association and its binding to the nucleic acid has already been observed in other systems such as the Arc repressor and its operator DNA (Silva & Silveira, 1993) and the R17 bacteriophage when compared to its coat protein dimer (Da Poian *et al.*, 1993). This kind of coupling seems to be very important to biological events such as the recognition of the nucleic acid by the repressors or inducers and the assembly of protein/nucleic acid complexes.

The reversibility of the pressure disassembly of ribonucleoprotein particles of CPMV implies that the assembly occurs at a very low protein concentration when the RNA is present. This property of the interaction between the proteins and the RNA could be related to a protection of the RNA against the degradation by cell ribonucleases. In fact, in the absence of RNA 2 (which codes for the coat proteins), RNA 1 accumulates to a much lower level than it does in a full infection (de Varennes & Maule, 1985), probably because of its increased turnover when unprotected by the proteins. On the other hand, the irreversibility of the dissociation of the top component leads to the question of how empty capsids can be formed *in vivo*. As described by Bruening (1969), a large excess of coat proteins synthesized during infection could promote the assembly of the empty shells. Bruening also found that a strain of CPMV that had a mutation in the middle-component RNA was unable to produce the top component. An attractive possibility is that a specific RNA sequence may act by helping in the assembly of a subparticle, for example, a pentameric unit, and then release this unit to act in the assembly of others from the capsid proteins. This hypothesis would explain both the lower pressure stability and the absence of reassembly of the empty shells.

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