Cold Denaturation of an Icosahedral Virus. The Role of Entropy in Virus Assembly†

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ABSTRACT: Assembly of icosahedral viruses is not completely understood at the molecular level. The main puzzle is to answer how chemically identical protein subunits take up unique positionally dependent conformations during the process of assembly. The stability of the ribonucleoprotein particles of cowpea mosaic virus (CPMV) to pressures and subzero temperatures has been studied. At room temperature, reversible pressure denaturation of CPMV is obtained only in the presence of 5.0 M urea. On the other hand, when the temperature is decreased to −15 °C, the ribonucleoprotein components denature, at 2.5 kbar, in the presence of 1.0 M urea. At temperatures close to −20 °C, denaturation is obtained even in the absence of urea. Whereas the denaturation promoted by pressure and urea at room temperature is reversible, virus particles denatured when the temperature is decreased under pressure cannot reassemble. Bis-ANS binding data suggest that this irreversibility may be related to protein release from RNA, which probably does not occur under denaturating conditions at room temperature. The contributions of enthalpy (ΔH^*) and entropy (ΔS^*) for the free energy of association of CPMV are calculated from the cold denaturation curves under pressure. The entropy change is positive and large, making the assembly of ribonucleoprotein components an entropy-driven process, suggesting that the burial of nonpolar side chains during the process of assembly is the structural foundation for CPMV assembly.

Virus assembly involves the questions of protein folding, protein assembly, and nucleic acid recognition. Understanding the mechanism by which the plasticity required for successful virus assembly is coded into the folded conformation of the capsid protein subunit is necessary to assess its thermodynamics. It can be deduced that a coupling exists between the conformational changes in the coat protein and assembly of the virion. This coupling can be approached by equilibrium and kinetic studies on the stability of the virus particles. In recent years, crystallographic analyses of spherical RNA viruses have revealed capsid structures at near-atomic resolution (Rossmann & Johnson, 1989). However, the thermodynamics and the mechanism of virus assembly are still far from completely understood. Hydrostatic pressure has been used to promote dissociation and denaturation of oligomeric proteins (Weber & Drickamer, 1983; Silva & Weber, 1993) as well as viral structures (Silva & Weber, 1988; Silva *et al.*, 1992*a*; Da Poian *et al.*, 1993, 1994), and it has been successfully utilized to analyze the role of nucleic acid in stabilizing subunit interactions in oligomeric proteins and viral capsids (Silva & Silveira, 1993).

Cowpea mosaic virus (CPMV) is a comovirus that has a bipartite, single-stranded, positive-sense RNA genome. The CPMV capsids contain equimolar amounts of a large (L) and a small (S) coat protein, with molecular weights of 42 000 and 23 000, respectively (Wu & Bruening, 1971). The two RNA molecules are encapsidated into separate virus particles. Empty shells are also formed *in vivo*, resulting 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. A preliminary crystallographic 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). For bean pod mottle virus (BPMV), another member of the comovirus family, well-ordered RNA interacting with each asymmetric unit of the particle was detected for the first time in an X-ray diffraction map (Chen *et al.*, 1989). An analysis of these data made possible the construction of a model for RNA packing in the BPMV middle component (Chen *et al.*, 1990).

The stabilization conferred on the CPMV capsid by RNA has been measured by comparing pressure−disassembly of empty and ribonucleoprotein particles at room temperature (Da Poian *et al.*, 1994). Although the top component could be denaturated by pressure in the presence of 1.5 M urea, pressure denaturation of the ribonucleoprotein particles was obtained only in the presence of 5.0 M urea. We use the general term "pressure denaturation" to refer to the spectral shift of tryptophan emission. This, however, does not mean that there was complete unfolding. We have utilized here subzero temperatures to promote denaturation of this virus. A number of proteins exhibit cold denaturation or cold inactivation (Privalov, 1990; Brandts, 1964; Privalov *et al.*, 1986; Griko *et al.*, 1988; Chen & Schellman, 1989). In fact,

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one of the first observations on cold dissociation was the description by Lauffer and collaborators (Lauffer *et al.*, 1958) that the reversible dissociation of the capsid subunits of tobacco mosaic virus was facilitated by the decrease in temperature. However, most proteins do not cold-denature at temperature ranges higher than 0 °C. Since at 2.5 kbar the freezing point of water is shifted to approximately −20 °C, we can utilize high pressure to reach subzero temperatures while maintaining an aqueous system in the liquid state. The dissociation and denaturation of phycobilisomes (Foguel *et al.*, 1992), allophycocyanin (Foguel & Weber, 1994), Arc repressor−operator DNA complex (Foguel & Silva, 1994), and the coat protein shells of bacteriophage P22 (Prevelige *et al.*, 1994) by hydrostatic pressure at subzero temperatures has made it possible to examine the cold denaturation phenomenon under conditions of equilibrium in dilute aqueous buffer solutions of the protein.

MATERIALS AND METHODS

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.

CPMV Samples. The Bil mutant of the yellow strain of cowpea mosaic virus was kindly supplied by Dr. John E. Johnson. It was purified as described elsewhere (Siler *et al.*, 1976; White & Johnson, 1980) and separated into components by equilibrium centrifugation in a self-forming 40% cesium chloride gradient at pH 7.0 (Schmidt *et al.*, 1983). The protein concentration utilized in all the experiments was 50 μg/mL.

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 the dissociated and associated forms, respectively.

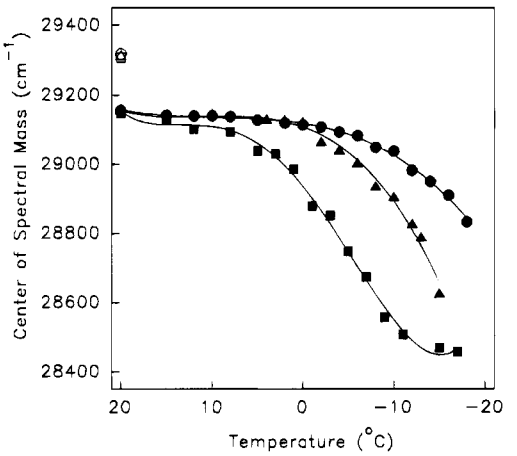

FIGURE 1: Cold denaturation of the CPMV bottom component under pressure. The effects of temperature decrease on the center of spectral mass of the bottom component were measured at 2.5 kbar in the absence (●) and in the presence of 0.5 (▲) and 1.0 M urea (■). The empty symbols correspond to the respective values before pressure application. The standard deviations are smaller than the symbols used.

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 the fluorescence at 330 nm (excitation at 280 nm) and the absorbance at 260 nm.

RESULTS AND DISCUSSION

Cold Denaturation of Cowpea Mosaic Virus. The effects of temperature decrease on the tryptophan fluorescence emission spectrum of the CPMV bottom component were investigated in the presence of 0−1.0 M urea, at 2.5 kbar (Figure 1). In the absence of urea, 50% of the spectral change was obtained only at −18 °C. On the other hand, complete virus denaturation was observed at this temperature when the virus was incubated with 1.0 M urea. At the urea concentration range utilized in these experiments, almost no effects were observed when pressure was applied at room temperature. At 1.0 M urea, the tryptophan fluorescence spectrum underwent a large red shift when the temperature was decreased, at 2.5 kbar (Figure 2A, top). At −15 °C, complete denaturation of the sample shown in the Figure 2A was confirmed by measuring light scattering, which decreases as the particles dissociate (Figure 2B, middle). The curves relating the center of spectral mass and light scattering to temperature are practically superimposed. On the contrary, after return to room temperature and pressure release, the center of spectral mass underwent a blue shift, whereas the light-scattering value remained low (symbols in parentheses of Figure 2B). This result may be interpreted as conformational changes or limited oligomerization of the coat proteins without formation of a capsid.

In order to obtain the apparent thermodynamic parameters of CPMV assembly, a van't Hoff plot was constructed (Figure 2C, bottom) from the disassembly curve shown in Figure 2B. It should be pointed out that this procedure is limited by the fact that the cold denaturation of CPMV is irreversible and therefore we designate the parameters as apparent values

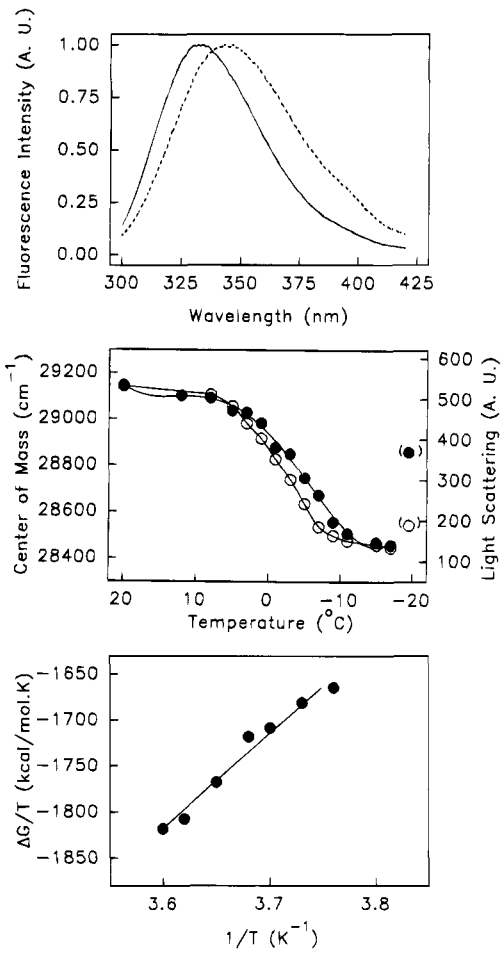

FIGURE 2: Cold disassembly of the bottom component in the presence of 1.0 M urea. (A, top) Tryptophan fluorescence spectra at 2.5 kbar, at 20 °C (—) and at −15 °C (– – –). (B, middle) Disassembly curves obtained by measuring the center of spectral mass of tryptophan emission (●) and the light scattering (○). The symbols *in parentheses* correspond to the values after pressure release. The standard deviations are smaller than the symbols used. (C, bottom) van't Hoff plot constructed from the fluorescence curve of (B).

(ΔG^*, ΔH^*, and ΔS^*). Since we were dealing with an irreversible process, we have used a steady-state approach (Denbigh, 1951; de Groot, 1952; Yourgrau *et al.*, 1966; Lavenda, 1978) for the thermodynamic treatment of our data. Assuming the reaction scheme:

$$(LS)_{60}R \underset{k_{-1}}{\overset{k_{+1}}{\leftrightarrow}} 60(LS)R \xrightarrow{k_2} 60(LS) + R$$

where $LS_{60}R$ is the ribonucleoprotein particle, (LS)−R is the denatured capsid protein (large and small subunits) bound to RNA, (LS) and R are irreversibly denatured subunits and free ribonucleic acid, respectively (Da Poian *et al.*, 1994), and k_{+1}, k_{-1}, and k_2 are rate constants. A steady state seems to develop where the denaturation reaction—detected by formation of protein species with red-shifted tryptophan emission—does not change with time, at least during the time scale of the reaction (minutes to hours). Therefore:

$$k_{+1}[(1 - \alpha)C] = (k_{-1} + k_2)(60\alpha C)^{60} \qquad (3)$$

where C is the molar concentration expressed in terms of whole virions and α is the degree of dissociation at a given temperature. Thus, the apparent dissociation constant at a temperature T (K_T^*) can be derived:

$$K_T^* = \frac{k_{+1}}{k_{-1} + k_2} = \frac{60^{60}C^{59}\alpha^{60}}{1 - \alpha} \qquad (4)$$

This equation is similar to the dissociation constant determined for reactions at equilibrium or close to equilibrium (Silva & Weber, 1993; Borafe *et al.*, 1994). The apparent free energy (ΔG^*) is calculated from the dissociation constant (KT^*) at a given temperature:

$$\Delta G^* = RT \ln K_T^* \qquad (5)$$

The van't Hoff plot is based on a rearrangement of the Gibbs relation ($\Delta G^* = \Delta H^* - T\Delta S^*$), and shows the change in enthalpy and entropy from the slope (ΔH^*) and the intercept on the ordinate ($-\Delta S^*$). The asterisks indicate that these are steady-state, rather than equilibrium values.

Denaturation of CPMV Is Mostly Due to a Decrease in Entropy. Table 1 summarizes the parameters obtained in the presence of different urea concentrations expressed in kilocalories per mole. For all the conditions, the end points were assumed to be the same based on the fact that the value of the center of spectral mass (28 450 cm^{-1}) corresponds to a limiting value of exposure of tryptophan to the aqueous medium found in pressure-denatured ribonucleoprotein and empty particles (Da Poian *et al.*, 1994). If virus dissociation is facilitated by decreasing temperature, the assembly of the shells is expected to be an entropy-driven process. As found before for oligomeric proteins (Weber, 1993), the association of CPMV is related to large and positive variations in enthalpy and entropy. The entropy change is larger than the enthalpy variation, indicating that maintenance of CPMV ribonucleoprotein particles assembled is the result of an entropy-driven process. These data support the view that assembly involves conformational changes characterized mainly by a decrease in the contacts of nonpolar side chains with water (increase in "hydrophobic bonds"). However, in addition to the apolar interactions, other weak bonds may be involved in stabilizing the assembled state such as hydrogen bonds, dipole−dipole-induced interactions, and the ionization state of some side chains. In a well-studied system, the tobacco mosaic virus, Lauffer's group has studied the contribution of different forces to make the assembly of the particle driven by entropy [for reviews, see Lauffer (1975), Shalaby and Lauffer (1983, 1985), and Lauffer and Shalaby (1985)]. Recently, Privalov and Makhatadze (1993) have pointed out that the hydration of polar residues should also result in a decrease in entropy. For a simpler protein, Arc repressor, the entropy-driven folding and association is followed by displacement of solvent (Foguel & Silva, 1994), and the volume change decreases linearly as water is replaced with glycerol (Oliveira *et al.*, 1994), reflecting that several nonpolar side chains are exposed to the solvent in the denatured state and are buried in the native dimer. Recently, Weber (1993) has proposed an alternative explanation for the entropy-driven condensation of proteins. Weber proposes that the entropy-driven association of protein subunits would be explained by the entropy change associated with the conversion of protein−water bonds into protein−protein bonds, which are weaker. In this view, the normal associated

Table 1: Apparent Enthalpy (ΔH^*) and Apparent Entropy ($T\Delta S^*$) Contributions to the Free Energy of Association of CPMV Bottom Component at 2.5 kbar, Calculated for $T = 20$ °C[a]

[urea] (M)	DH*	$T\Delta S^*$	ΔG^*	$\Delta H^*/n$	$T\Delta S^*/n$	$\Delta G^*/n$
0	830 ± 75	1458 ± 85	−628.2	13.8 ± 1.25	24.3 ± 1.42	−10.5
0.5	1005 ± 43	1628 ± 48	−622.5	16.8 ± 0.77	27.1 ± 0.80	−10.4
1.0	1027 ± 75	1616 ± 81	−588.8	17.1 ± 1.25	26.9 ± 1.35	−9.8
2.5	1217 ± 22	1799 ± 24	−582.6	20.3 ± 0.37	30.0 ± 0.40	−9.7

[a] The thermodynamic parameters are nonequilibrium values and are expressed in kilocalories per mole. The temperature is expressed in degrees kelvin. We considered $n = 60$ (Da Poian *et al.*, 1994).

state of a protein is a result of the formation of a considerable number of weak protein−protein bonds. The classical theory of hydrophobic bond (Kauzmann, 1959) and Weber's theory equally explain the cold denaturation of proteins. Both Privalov and Weber recognize that the participation of van der Waals interactions to keep the native structure of proteins has been underestimated.

Reversibility vs Irreversibility in the Pressure Denaturation of CPMV. The ability of denatured virus to reassemble was evaluated using size-exclusion high-performance liquid chromatography. In a previous report, it was observed that at room temperature and 2.5 kbar, both bottom and middle components dissociate completely only in the presence of 5.0 M urea (Da Poian *et al.*, 1994). The elution pattern of these samples after pressure release showed that the dissociated viruses could reassemble into capsids. Both native virus and pressurized virus were detected 8 min after injection, close to the void volume of the column (Da Poian *et al.*, 1994). On the contrary, cold-denatured ribonucleoprotein particles cannot reassemble, as shown by the experiments of Figure 3. The elution of the CPMV bottom component sample in the presence of 1.5 M urea occurred 8 min after injection, in the same position as the native virus, as detected by RNA absorption at 254 nm and protein fluorescence emission at 330 nm (Figure 3A). When this sample was pressurized at 2.5 kbar and subjected to a temperature of −10 °C and then returned to room temperature and atmospheric pressure, a heterogeneous peak of absorption at 254 nm was detected (solid line of Figure 3B). This peak corresponds to the RNA molecule with practically no bound protein, since very little fluorescence (dashed line) was detected in the elution of this sample. The denatured coat proteins adsorb to the column gel, since they were detected in the solution by fluorescence before injection (not shown). We have previously found that the disassembled top component also adsorbs to this column, being undetectable by both fluorescence at 330 nm and protein absorption at 280 nm, although the native top component elutes at the same position as the native ribonucleoprotein particles (Da Poian *et al.*, 1994). While the sample of Figure 3A was not affected by incubation with ribonuclease (Figure 3C), almost all the RNA was digested when the sample that was pressurized and subjected to −10 °C was incubated with that enzyme: the major peak of absorption at 254 nm appeared in the final volume of the column (Figure 3D). These data suggest that the protein−RNA interactions are disrupted irreversibly when the virus is subjected to low temperatures under pressure.

To investigate why cold denaturation of CPMV ribonucleoprotein particles is an irreversible process while pressure denaturation is not, we utilized a fluorescent probe, bis(8-anilinonaphthalene-1-sulfonate) (bis-ANS), which binds noncovalently to apolar domains of proteins. Bis-ANS binding

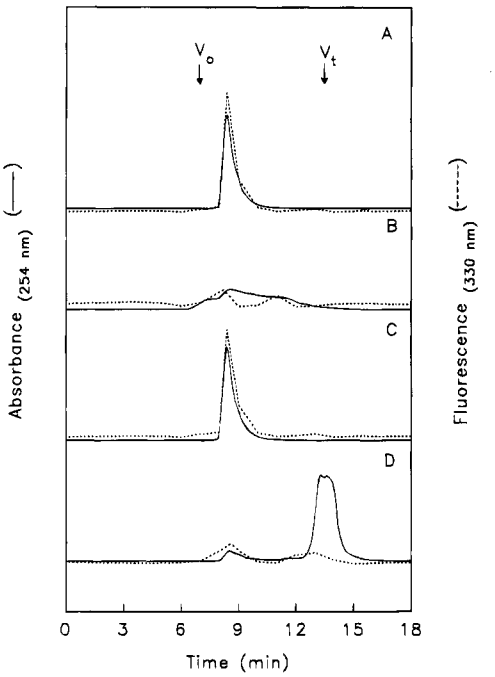

FIGURE 3: High-performance liquid chromatography. (A) Elution profile of native CPMV bottom component in the presence of 1.5 M urea, at room temperature. (B) The virus was pressurized at 2.5 kbar and −10 °C for 1 h in the presence of 1.5 M urea and injected in the column after return to room temperature and atmospheric pressure. (C, D) Samples from (A) and (B), respectively, were then incubated for 30 min with ribonuclease (2 μg/mL final concentration) and injected in the column. The continuous lines correspond to the absorbance measurement at 254 nm, and the dotted lines correspond to the fluorescence measurement (excitation at 280 nm and emission measured at 330 nm).

is accompanied by a large increase in its fluorescence quantum yield and has been used to probe protein structural changes (Rosen & Weber, 1969; Horowitz *et al.*, 1984; Silva *et al.*, 1992*b*). On the basis of the increase in fluorescence, bottom CPMV coat proteins gradually bound more bis-ANS as the temperature was decreased under pressure (Figure 4A, top panel). Nevertheless, while cold denaturation of the virus promotes the exposure of protein domains that bind the probe, the RNA-containing particles disassembled by pressure and 5.0 M urea, at room temperature, did not bind bis-ANS (Figure 4B, bottom panel). An interpretation for this result would be a strong interaction between proteins and RNA after breakage of the protein−protein contacts by pressure at room temperature, what could explain the reversibility of the process found for this condition (Da Poian *et al.*, 1994) (Figure 5). The capsid proteins seem to continue binding the RNA, preventing the binding of bis-ANS. On the other hand, the conjoint action of pressure and subzero temperature probably leads the coat proteins to release the nucleic acid, which would make the cold denaturation an irreversible process.

Table 2: Comparison of Pressure and Cold Denaturation of Ribonucleoprotein CPMV Components[a]

	pressure denaturation (5.0 M urea)		cold denaturation (1.5 M urea)	
	center of mass (cm^{-1})	light scattering (AU)	center of mass (cm^{-1})	light scattering (AU)
		Bottom Component		
native	29058.8	3603.0	29117.2	3628.9
denatured	28187.0	2454.9	28135.9	664.1
return	28960.6	3278.5	28697.3	1217.7
		Middle Component		
native	29061.0	3587.3	29082.8	3833.2
denatured	28119.5	2704.1	28148.6	632.0
return	28924.2	3256.8	28740.3	1218.1

[a] The errors for the values of the center of spectral mass are lower than 10 cm^{-1}, and for light scattering, values are lower than 1.5%.

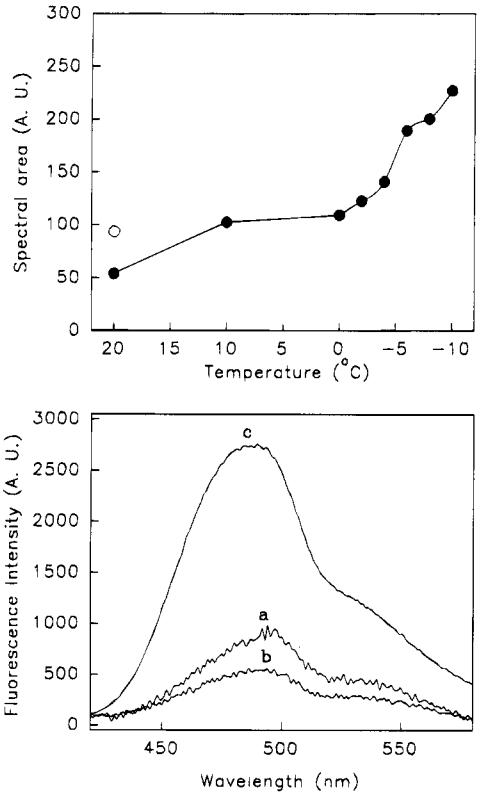

FIGURE 4: (A, top) Bis-ANS binding to the CPMV bottom component. Effects of low temperature on the area of bis-ANS fluorescence spectra. CPMV bottom component (50 μg/mL) was incubated with bis-ANS (2 μM final concentration) at atmospheric pressure in the presence of 1.5 M urea (○). Then, the sample was pressurized to 2.5 kbar, and the temperature was gradually decreased (●). The standard deviations are smaller than the symbols used. (B, bottom) Bis-ANS fluorescence spectra obtained when the probe was incubated with native (a) and pressure-denatured (b) and cold-denatured (c) bottom CPMV. In (b), the denaturation was obtained by incubation with 5.0 M urea and increasing the pressure to 2.5 kbar, at room temperature. In (c), denaturation was obtained by applying pressure in the presence of 1.5 M urea and decreasing the temperature to −10 °C. The excitation wavelength was 360 nm, and the emission was measured from 420 to 580 nm. A.U. = arbitrary units.

Further evidence for this hypothesis is provided by light-scattering measurements on pressure and cold-denatured CPMV (Table 2). Although tryptophans were completely exposed to the medium during pressure application in the presence of 5.0 M urea, at room temperature (the center of spectral mass was completely red-shifted), the light-scattering value decreased by only 30%. This finding indicates that the virus was denatured into large particles, as expected if the proteins remained bound to the RNA. This could explain

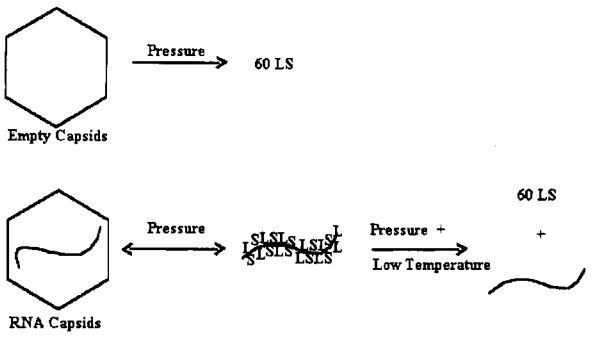

FIGURE 5: Schematic representation of the dissociation of empty particles by pressure (top) and of ribonucleoprotein particles by pressure and by pressure and subzero temperatures (bottom). In the case of the RNA capsids, pressure promotes breakage of the structure, but the capsid proteins (L and S) still remain bound to the RNA, and the reaction is fully reversible. Cold denaturation under pressure leads to capsid protein dissociated from the RNA and loss of reversibility.

why disassembly of the bottom and middle components is a reversible process while the top component (lacking RNA) can not reassemble after denaturation by pressure (Da Poian et al., 1994) (see schematic representation in Figure 5). On the other hand, light scattering of cold-denatured ribonucleoprotein particles decreased approximately 6-fold, consistent with the release of the RNA and the formation of much smaller particles. After cold denaturation, the light scattering did not recover the initial value when temperature and pressure were restored to control conditions (Figure 5). This irreversibility was also shown by chromatography (Figure 3).

CONCLUSIONS

The data described above suggest that low temperatures affect both protein−protein and protein−RNA interactions, showing that CPMV association can be seen as an entropy-driven process and that protein binding to RNA may be essential to virus assembly. Pressure dissociation studies have shown that a DNA binding protein, Arc repressor, is strongly stabilized by DNA operator (Silva & Silveira, 1993). Similarly, the coat protein dimer of R17 bacteriophage is less stable than the whole phage (Da Poian *et al.*, 1993). The complex Arc repressor−operator DNA is also cold-denatured at subzero temperatures under pressure, demonstrating that the favorable entropy increases greatly when Arc repressor binds tightly its operator sequence but not a nonspecific sequence (Foguel & Silva, 1994). The procapsid shells of bacteriophage P22 only dissociate by pressure at temperatures below 0 °C whereas the monomeric coat protein is very unstable toward pressure (Prevelige *et al.*, 1994).

Application of the pressure methods used to promote dissociation, applied at subzero temperatures, has made information on the thermodynamics of protein folding, DNA recognition, and virus assembly. Some difficulties arise from the fact that there is a large coupling between kinetics and equilibrium in biological processes. As pointed out by Denbigh (1951), no observable bodies are ever entirely at equibrium but are subject always to small changes. The challenge of biochemical thermodynamics is to abstract from the complexity of the real biological systems and in its place to substitute with a more or less idealized situation that is more amenable to analysis. Our apparent values of enthalpy and entropy try to assess the thermodynamics of virus assembly with these limitations.

At a molecular level, the cold denaturation of CPMV particles indicates that the reversal process, capsid assembly, is followed by burial of a large proportion of nonpolar side chains. Against expectation, the interactions that lead to CPMV assembly are entropy-driven, which means that they are dominated by weak interactions (nonpolar) and less contribution of strong interactions which would normally be expected to be driven by changes in ΔH. The cold-denatured capsid subunits apparently expose the 14 tryptophan side chains of the large and small subunits and the hydrophobic regions that bind bis-ANS. Therefore, the virus particle can be pictured as a giant protein and its constitutive capsid subunits as partly-unfolded domains that under specific conditions (chaperoned by RNA) undergo folding. The reduced stability of virus particles at high pressure and low temperature suggests the potential application to inactivate viruses of medical importance. We have already found that several complex viruses are inactivated by pressures at room temperature (Silva *et al.*, 1992a; Silva, 1993).

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