Tobacco Mosaic Virus Disassembly by High Hydrostatic Pressure in Combination with Urea and Low Temperature[†,‡]

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ABSTRACT: We investigated the effect of low temperature and urea combined with high pressure on tobacco mosaic virus (TMV). The evaluation of its aggregation state and denaturation process was studied using gel filtration, transmission electron microscopy, and spectroscopic methods. The incubation at 2.5 kbar induced 18% dissociation, and decreasing of temperature to −19 °C promoted additional dissociation to 72%, with stabilization of the dissociation products. Under such conditions, extensive denaturation did not occur. The apparent enthalpy and entropy of dissociation (ΔH^*_{dis} and $T\Delta S^*_{dis}$) were −9.04 kcal/mol subunit and −15.1 kcal/mol subunit, respectively, indicating that the TMV association is an entropicly driven process. The apparent free energy of stabilization given by the presence of RNA is at least −1.7 kcal/mol subunit. Urea-induced dissociation of TMV samples and incubation at high-pressure promoted a higher degree of dissociation. The volume change of dissociation decreased in magnitude from −16.3 to −3.1 mL/mol of dissociated subunit, respectively, in the absence and presence of 2.5 M urea, suggesting exposure of the protein−protein interface to the solvent. High-pressure induced remarkable TMV denaturation in the presence of 2.5 M urea, with a volume change of −101 mL/mol of denatured subunit. The apparent enthalpy and entropy of denaturation (ΔH^*_{den} and $T\Delta S^*_{den}$) by 1.75 M urea at 2.5 kbar was −11.1 and −10.2 kcal/mol subunit, respectively, demonstrating that the TMV protein coat presents an apparent free energy of denaturation by urea close to zero. Although the processes could not be assumed to be pure equilibria, these thermodynamic parameters could be derived by assuming a steady-state condition.

Tobacco mosaic virus (TMV)[1] is a rod-shaped virus, 3000 Å long and 180 Å in diameter. Approximately 2130 identical protein subunits of molecular weight of 17 500 form a helix, protecting a single 6.4 kb RNA molecule. The refined structure of TMV at 2.9 Å resolution has been described (*1*). This virus is a much studied model of subunit assembly and disassembly (*2*). Despite the large number of studies of this virus, at this moment, the thermodynamics of the TMV assembly and denaturation are not well understood. High hydrostatic pressure is an excellent tool to investigate protein−protein interactions since pressures up to 2.5 kbar induce significant dissociation of oligomeric proteins and viruses with few direct effects on protein conformation (*3*).

Some decades ago, Lauffer and Dow (*4*) studied the effect of pressure on TMV, observing at 7.5 kbar a pressure inactivation in minutes, with precipitation of a material depleted in nucleic acid after longer periods. Latter studies with the TMV protein coat suggested reversible depolymerization induced by decreasing temperature, with decreasing entropy (*5*). The direct effect of high pressure on the TMV protein coat demonstrated significant dissociation of helical rods and double disks at 1.5 kbar (*6*).

The pressure-induced dissociation of proteins follows the following thermodynamic relation:

$$K_p = K_{atm} \exp(p\Delta V^0_{dis}/RT) \quad (1)$$

where p is the pressure, ΔV^0_{dis} is the volume change of dissociation, K_p and K_{atm} are the equilibrium dissociation constants at pressure p and atmospheric pressure, respectively, R is the universal gas constant, and T is the absolute temperature.

The logarithmic form of this equation:

$$\ln K_p = \ln K_{atm} + p\Delta V^0_{dis}/RT \quad (2)$$

allows the calculation of K_{atm} and ΔV^0_{dis} through a plot of ln K_p as a function of pressure (*7*).

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[‡] We dedicate this paper to the memory of Professor Gregorio Weber.

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[1] Abbreviations: bis-ANS, bis-(8-anilinonaphthalene-1-sulfonate); TMV, tobacco mosaic virus; BMV, brome mosaic virus; CPMV, cowpea mosaic virus.

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In the present study, we investigated the dissociation of TMV by pressure in the presence of urea and at subzero temperatures. The virus presented structural stability at 2.5 kbar, which was eliminated in the presence of subdenaturing concentrations of urea or at subzero temperatures. Under these latter conditions, there was no detectable denaturation. The apparent thermodynamic parameters of dissociation and denaturation were analyzed.

EXPERIMENTAL PROCEDURES

Chemicals. All reagents were of analytical grade. Distilled water was filtered and deionized through a Millipore water purification system to 18 MΩ resistance. Unless stated otherwise, the experiments were performed in the presence of standard buffer 0.05 M tris chloride, in 0.150 M NaCl, pH 7.5.

Methods. TMV Purification. The virus was isolated from Turkish tobacco plants infected with the common strain of the virus. The purification method was described previously (*8*).

Light-Scattering and Fluorescence Studies under Pressure. The high-pressure bomb has been described previously (*9*). Light-scattering and fluorescence spectra were recorded on a SLM Aminco SPF-500C spectrofluorometer (Urbana, IL).

Light scattering at 340 nm was measured at 90° in the spectrofluorometer by selecting the same wavelengths for both the excitation and emission monochromators. The degree of dissociation α was related to the intensity of light scattering at pressure p, S_p, by

$$\alpha_p = (S_i - S_p)/(S_i - S_f) \quad (3)$$

were S_f and S_i are the intensities of light scattering for dissociated and associated forms, respectively.

Fluorescence spectra at pressure p were calculated by the specification of the center of mass (ν_p).

$$\nu_p = \sum_i \nu_i F_i / \sum_i F_i \quad (4)$$

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 degree of denaturation at pressure p, $\alpha_{den,p}$, is related to ν_i by the expression

$$\alpha_{den,p} = [1 + Q(\langle\nu_p\rangle - \langle\nu_{des}\rangle)/(\langle\nu_n\rangle - \langle\nu_p\rangle)]^{-1} \quad (5)$$

where Q is the ratio of the quantum yields of denatured and native forms, ν_p is the center of mass at pressure p, and ν_{des} and ν_n are the corresponding quantities for denatured and native forms (*10*).

Extrinsic fluorescence studies were performed by using bis-ANS, and the emission spectra were taken with λ_{ex} = 360 nm and λ_{em} = 420−650 nm.

Size-Exclusion High-Performance Liquid Chromatography. High-performance liquid chromatography gel filtration was performed in a prepacked SynChropack GPC 1000 column of 250 × 4.6 mm i.d., obtained from SynChrom, Inc. (Linden, IN). A Hewlett-Packard series 1050 system was utilized. Typically, a flow rate of 0.3 mL/min was used. Elution of the sample was monitored by absorption at 280 nm. The void volume (V_0) of the column was measured with purified TMV and the total volume (V_t) with a solution of albumin.

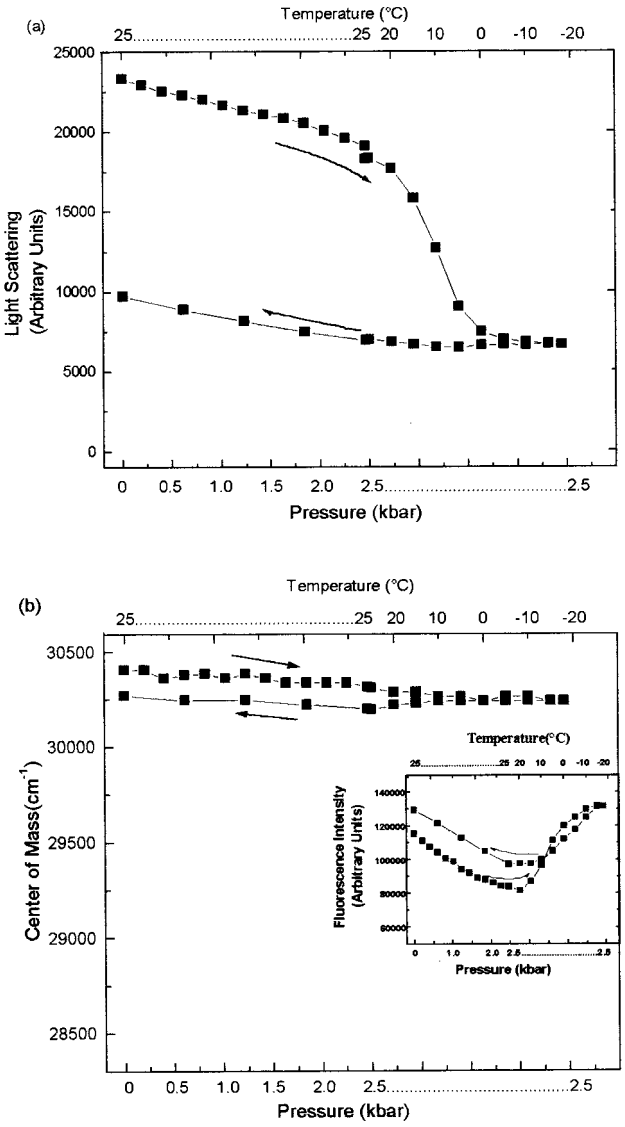

FIGURE 1: Effect of pressure and low temperature on light-scattering and fluorescence properties of TMV. A solution with a viral concentration of 1.0 mg/mL in Tris-Cl buffer, pH 7.5, and 150 mM NaCl was subjected to high pressure, to low temperature at 2.5 kbar and, later, returned to 25 °C and atmospheric pressure. Each spectroscopic data was collected 5 min after the pressure or temperature value had stabilized. The average interval of time needed for the temperature decrease was 15 min, and for the temperature increase, it was but a few minutes. The standard deviations are smaller than the symbols used. (a) Light-scattering intensity at 90° of the incident light, λ_{ex} = 340 nm. (b) Center of mass of emission fluorescence spectra, calculated as indicated in the Materials and Methods, eq 4. λ_{ex} = 285 nm, λ_{em} = 300−400 nm. (Inset) Intensity of emission fluorescence spectra.

Electron Microscopy. Transmission electron microscopy was performed in a ZEISS CEM-902 equipment. Negative staining was performed with 1% uranyl acetate.

RESULTS

Effect of High Pressure and Low Temperature on TMV. Figure 1 shows the effect of pressure and low temperature

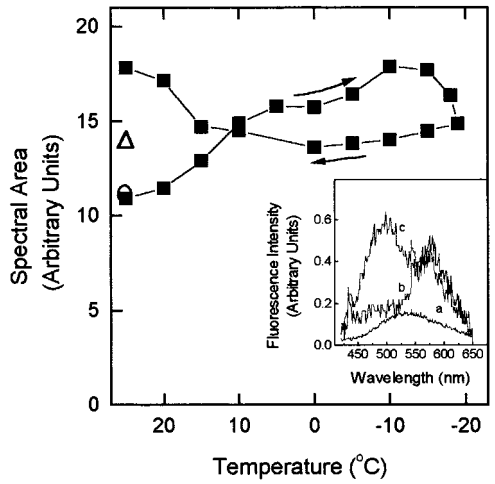

FIGURE 2: Effect of pressure and low temperature on the fluorescence of bis-ANS and TMV, $\lambda_{ex} = 360$ nm, $\lambda_{em} = 420-650$ nm. Open symbols indicate atmospheric pressure, before (○) and after (△) the pressure and temperature changes. The standard deviations are smaller than the symbols used. (Inset) Fluorescence emission spectra of 2 μM bis-ANS free in solution (a), or in the presence of TMV at atmospheric pressure and 25 °C (b), or at 2.5 kbar and −15 °C (c). Other conditions were as in Figure 1.

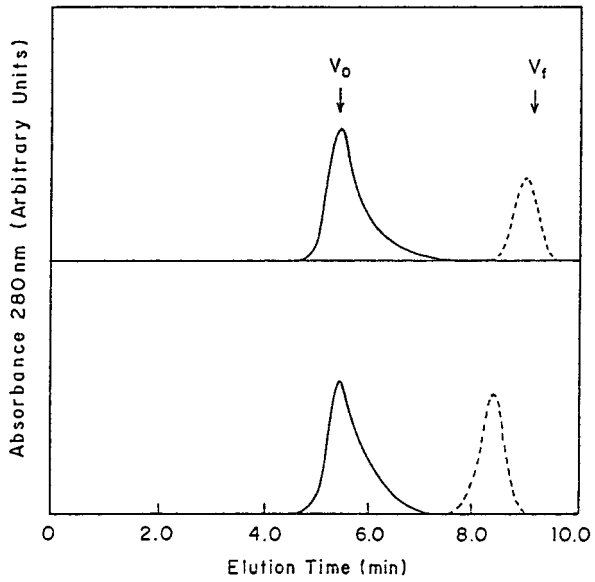

FIGURE 3: Gel filtration analysis of TMV (column Synchropack GPC 1000). (a) Virus incubation at atmospheric pressure (continuous line) and after incubation at 2.5 kbar and −18 °C, 1 or 20 days (dashed line). (b) Virus incubation in the presence of 2.5 M urea and atmospheric pressure for 2 days (continuous line), and 2 days after incubation at 2.5 kbar in the presence of 2.5 M urea (dashed line). V_0 and V_f correspond to dead and final volume of column, that are respectively the elution of native TMV (4×10^7 Da) and albumin (6.6×10^4 Da). Other conditions were as in Figure 1.

on a 1.0 mg/mL TMV solution in Tris-Cl buffer at pH 7.5. Light scattering showed a decrease of intensity with pressure and temperature, Figure 1a. The aggregation state of TMV was based on light-scattering intensity (see Materials and Methods) with degree of dissociation α corresponding to 0 for TMV at atmospheric pressure and α = 100% at 2.5 kbar in the presence of 6.0 M urea. A pressure of 2.5 kbar induced 18% TMV dissociation, and a temperature decrease to −19 °C at 2.5 kbar induced a further dissociation to 72%. The return of temperature to 25 °C and further return to atmospheric pressure induced poor recovery of light-scattering intensity, suggesting stabilization of dissociated products of TMV at least during the experimental observations. The reversibility was close to 70% when the virus solution was cooled to not less than 0 °C (data not shown) and, in the absence of a temperature change, the process was totally reversible (Figure 6a, upper curve, and Figure 4c).

Fluorescence studies indicated negligible red shift of emission spectra, Figure 1b, suggesting no significant denaturation even at conditions where a high degree of dissociation exists (−19 °C at 2.5 kbar). This spectroscopic parameter is very informative and is based on the exposure of aromatic residues to the bulk solvent in the denaturation process. The consequence is a red shift due to high dipole−dipole interactions between the excited molecule and the solvent, leading to a decrease in energy emission. Figure 1b, inset, shows the decreasing fluorescence intensity with pressure and further fluorescence intensity increasing as the temperature decreases. These results suggest that the partial dissociation induced by pressure exposes aromatic residues to solvent-promoting quenching through collisions with the solvent molecules. The lowering of temperature should decrease the collisional frequency.

The use of bis-ANS as an extrinsic probe showed no detectable changes of the fluorescence intensity with pressure up to 2.5 kbar, Figure 2. Further decreases in temperature promoted increases in the fluorescence signal. This can be due to more interactions between the probe and TMV through exposure of hydrophobic sites or positive residues of coat protein. Figure 2, inset, shows the emission fluorescence spectra corresponding to incubation of the sample at 2.5 kbar and −15 °C compared to the control, and of Bis-ANS in water.

Gel filtration chromatography in GPC 1000 of native TMV showed a single peak corresponding to a molecular mass above 10^7 Da, while incubation at 2.5 kbar and −15 °C and further return to 25 °C and atmospheric pressure (corresponding to the experiments of Figures 1 and 2) showed a peak of low molecular mass, Figure 3a.

Transmission electron microscopy images were in agreement with spectroscopic and gel filtration results. Figure 4a shows the purified TMV and Figure 4b the absence of viral structure after incubation at 2.5 kbar and −18 °C. The incubation of TMV at 2.5 kbar at 25 °C and return to atmospheric pressure, Figure 4c, also shows the presence of structures compatible with the native form of TMV. The incubation of TMV in the presence of 6.0 M urea at 2.5 kbar, Figure 4d, induced absence of viral structure as the incubation at low temperature.

As will be shown, the apparent volume change of dissociation ΔV^{0*}_{dis}, −16.3 mL/mol of subunit, can be calculated by plotting $\ln K^*_d$ as a function of pressure (Figure 7b, lower curve). The partial reversibility of TMV dissociation, at least on the observed time scale, indicates that a more appropriate methodology for calculation of the thermodynamic parameters should be considered. We have applied the steady-state approach (*11*−*14*) for the thermodynamics treatment of our data. Assuming the dissociation process of TMV as the following reaction:

$$P_{2130}G \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} 2130P + G \xrightarrow{k_2} 2130P^* + G \qquad (6)$$

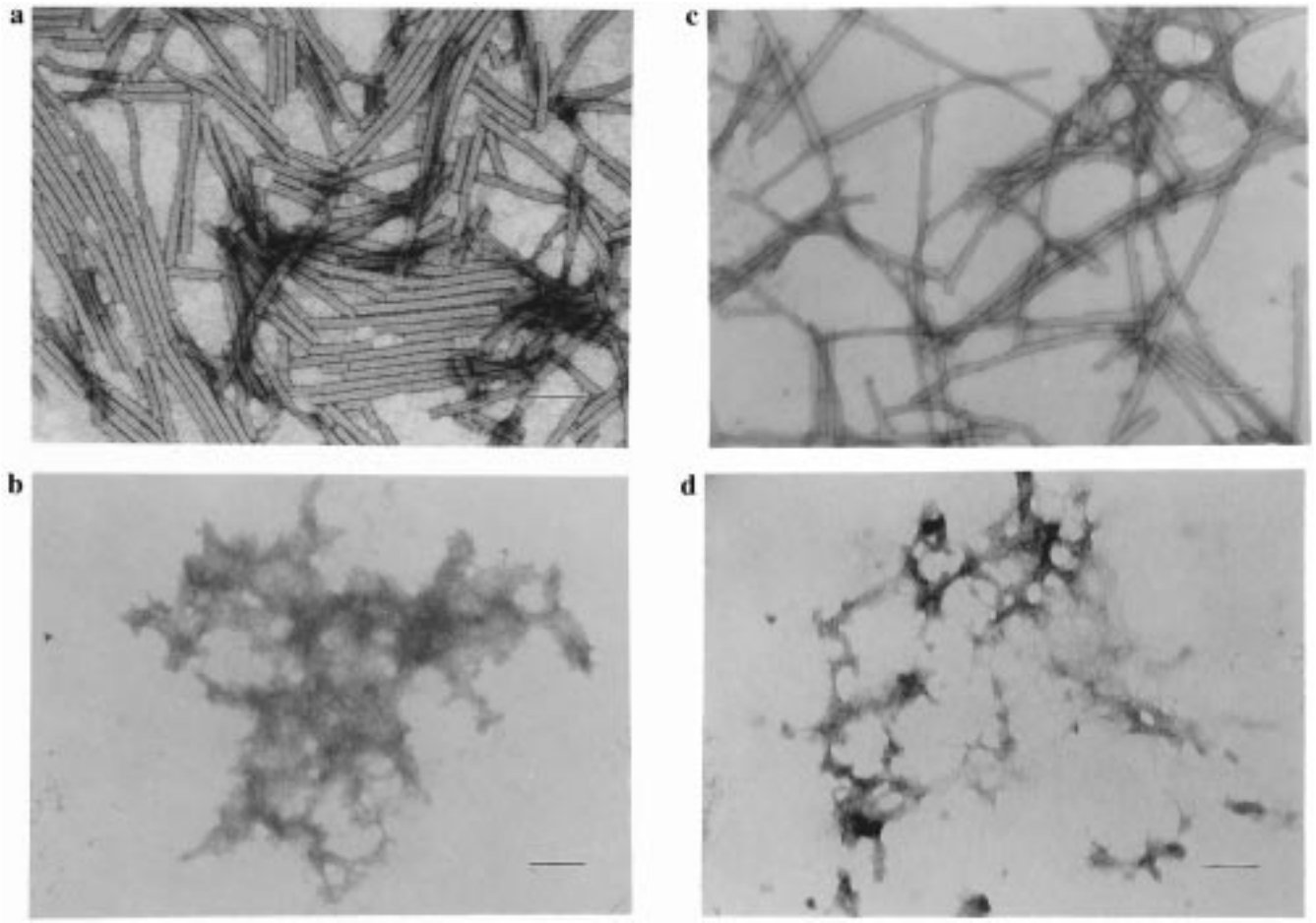

FIGURE 4: Transmission electron microscopy of TMV subjected to pressure and low temperature. TMV (1.0 mg/mL) (a) incubated at atmospheric pressure, (b) after incubation for 1 h at 2.5 kbar and −18 °C, (c) 1 day after 1 h incubation at 2.5 kbar and 25 °C, and (d) 6.0 M urea and subjected to 2.5 kbar for 1 h. Negative staining: 1% uranyl acetate. Other conditions as in Figure 1. The bar corresponds to 100 nm.

where the virion $P_{2130}G$ goes to a reversible dissociation to coat protein P and the genetic material (RNA) G and after to an irreversible step due to a deeper change in conformation of free subunits P*. This form should not be very different from the native one based on the negligible red shift of the emission fluorescence spectra, like the one observed in the presence of urea, as will be showed further. k_{+1}, k_{-1}, and k_2 are rate constants. The apparent steady state occurred for the dissociation process at least during the time scale of minutes to hours. The relationship of rate constants and degree of dissociation α is given by

$$k_{+1}[(1-\alpha)C] = (k_{-1} + k_2)(2130 \times \alpha C)^{2130}\alpha C \quad (7)$$

where C is the molar concentration of whole virions. Therefore, the apparent dissociation constant K_p^* at pressure p can be written as

$$K_p^* = k_{+1}/(k_{-1} + k_2) = 2130^{2130}C^{2130}\alpha^{2131}/(1-\alpha) \quad (8)$$

This consideration is similar to previous approach with cowpea mosaic virus (CPMV) (*14*) and similar to dissociation constants determined for reactions close to or at equilibrium (*3*, *15*). The apparent free energy of dissociation calculated from K_p^* became

$$\Delta G_{dis}^* = -RT \ln K_p^* \quad (9)$$

Similarly, we can consider viral dissociation by temperature at a given pressure as K_T^*, and the calculation of the apparent thermodynamic parameters is basically the same:

$$\Delta G_{dis}^* = -RT \ln K_T^* \quad (10)$$

The apparent enthalpy and entropy of dissociation, respectively ΔH_{dis}^* and ΔS_{dis}^*, can be derived based on the equation

$$\Delta G_{dis}^* = \Delta H_{dis}^* - T\Delta S_{dis}^* \quad (11)$$

or

$$\Delta G_{dis}^*/T = \Delta H_{dis}^*(1/T) - \Delta S_{dis}^* \quad (12)$$

with the asterisks indicating a steady-state condition instead of equilibrium state. Equation 12 allows the construction of a van't Hoff plot, Figure 5. The calculation of ΔH_{dis}^* and $T\Delta S_{dis}^*$ at 2.5 kbar gave, respectively, −9.04 and −15.1 kcal/mol subunit [$\Delta S_{dis}^* = -52.6$ cal/(K mol of subunit)]. Values of K_p^* corresponding to lower values of n result in similar numerical results for ΔH_{dis}^* and $T\Delta S_{dis}^*$ per mole of subunit, with no substantial deviation. As an example, we corroborate this for $n = 1000$, $K_p^* = 1000^{1000}C^{1000}\alpha^{1001}/(1-\alpha)$, which differs by less than 5% in ΔH_{dis}^* and $T\Delta S_{dis}^*$

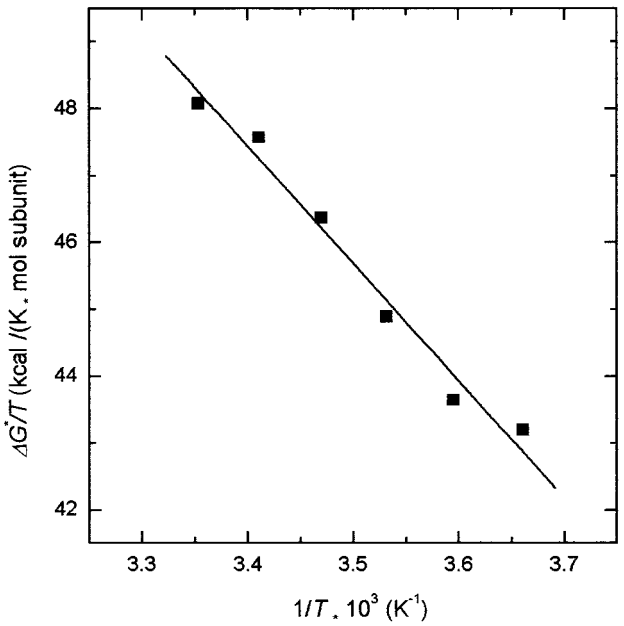

FIGURE 5: van't Hoff plot of TMV dissociation at 2.5 kbar based on the experiments of Figure 1a and eq 12. The values of the degree of dissociation were calculated from eq 3, considering $\alpha = 1$, the light-scattering intensity in the presence of 6.0 M urea and under 2.5 kbar. The standard deviations are smaller than the symbols used.

from the values obtained when $n = 2130$. Thus, even if the irreversible step occurs at lower values of n, the thermodynamic calculations are virtually unaltered.

Effect of High Pressure and Urea at Different Concentrations on TMV. The effect of urea on the TMV subjected to high pressure was followed by light-scattering and fluorescence studies (Figure 6). TMV at atmospheric pressure presented decreasing of light-scattering intensity at 1.0 M urea suggesting deaggregation even at subdenaturing concentrations and at 4.0 M reached a minimum light-scattering intensity corresponding to a high degree of dissociation (Figure 6a, inset). Figure 6a shows that pressure has induced dissociation at different urea concentrations (three upper curves). Since at 4.0 M urea and above the dissociation was near to 100%, there was no additional effect of pressure on the light-scattering intensity. The partial recovery of light-scattering intensity values indicates partial reversibility of the dissociation process.

Fluorescence detection showed that urea up to 2.5 M in concentration did not significantly change the center of mass of emission spectra of TMV (Figure 6b, inset), indicating very little denaturation. These results are expected since urea is usually subdenaturing at this concentration. An approximately maximum red shift in the fluorescence emission was reached at a urea concentration of 6.0 M. The high-pressure effect on the center of mass was especially sensitive in the experiment with 2.5 M urea, Figure 6b, showing clearly the shift from a near native form of coat proteins and partially dissociated TMV to a highly denaturated form. The partial recovery of center of mass values indicates a partial reversibility of TMV denaturation. Urea at higher concentrations induced a high degree of denaturation already at atmospheric pressure.

The partial reversibility of the denaturation process suggests a more appropriate approach considering a steady-state condition similar to a dissociation process. In this circumstance, the relationship of the apparent denaturation constant by pressure, $K^*_{\mathrm{den,p}}$, and the apparent volume change of denaturation, $\Delta V^{0*}_{\mathrm{den}}$, analogous to eq 2, is

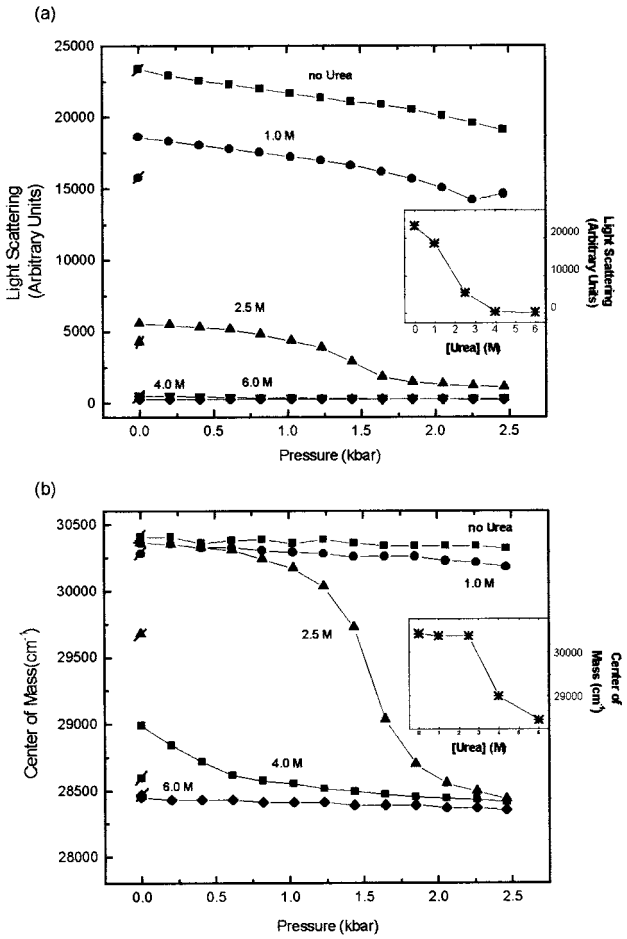

FIGURE 6: Effect of pressure and urea on light-scattering and fluorescence properties of TMV. The cut symbols correspond to effect of urea at atmospheric pressure. (■) Absence of urea and (●) 1.0, (▲) 2.5, (▼) 4.0 and (◆) 6.0 M urea. The standard deviations are smaller than the symbols used. (a) Light-scattering intensity detected as described in the Figure 1a, at different urea concentrations. (b) Center of mass of emission fluorescence spectra between 300 and 400 nm, $\lambda_{ex} = 285$ nm, calculated as indicated in the Materials and Methods, eq 4. Effect of urea concentration on TMV center of mass at atmospheric pressure. Other conditions as in Figure 1. Insets: Effect of urea at atmospheric pressure on light scattering (a) and on center of mass of emission fluorescence spectra (b).

$$\ln K^*_{\mathrm{den,p}} = \ln K^*_{\mathrm{atm}} + p\Delta V^{0*}_{\mathrm{den}}/RT \qquad (13)$$

The $\Delta V^{0*}_{\mathrm{den}}$ (eq 13) was calculated from the slope of Figure 7a, $\ln K^*_{\mathrm{den,p}}$ as a function of pressure, as -103.5 mL/mol subunit.

Analogously, the dissociation process is given by

$$\ln K^*_{\mathrm{p}} = \ln K^*_{\mathrm{atm}} + p\Delta V^{0*}_{\mathrm{dis}}/RT \qquad (14)$$

also based on eq 2. The apparent volume change of dissociation $\Delta V^{0*}_{\mathrm{dis}}$ and $\ln K^*_{\mathrm{atm}}$ can be calculated by plotting the logarithmic form of K^*_{p} versus pressure (Figure 7b). Table 1 shows $\Delta V^{0*}_{\mathrm{dis}}$ and $\ln K^*_{\mathrm{atm}}$ values calculated for the samples with urea concentrations up to 2.5 M. $\Delta V^{0*}_{\mathrm{dis}}$ decreases and $\ln K^*_{\mathrm{atm}}$ increases with the increase of urea concentration.

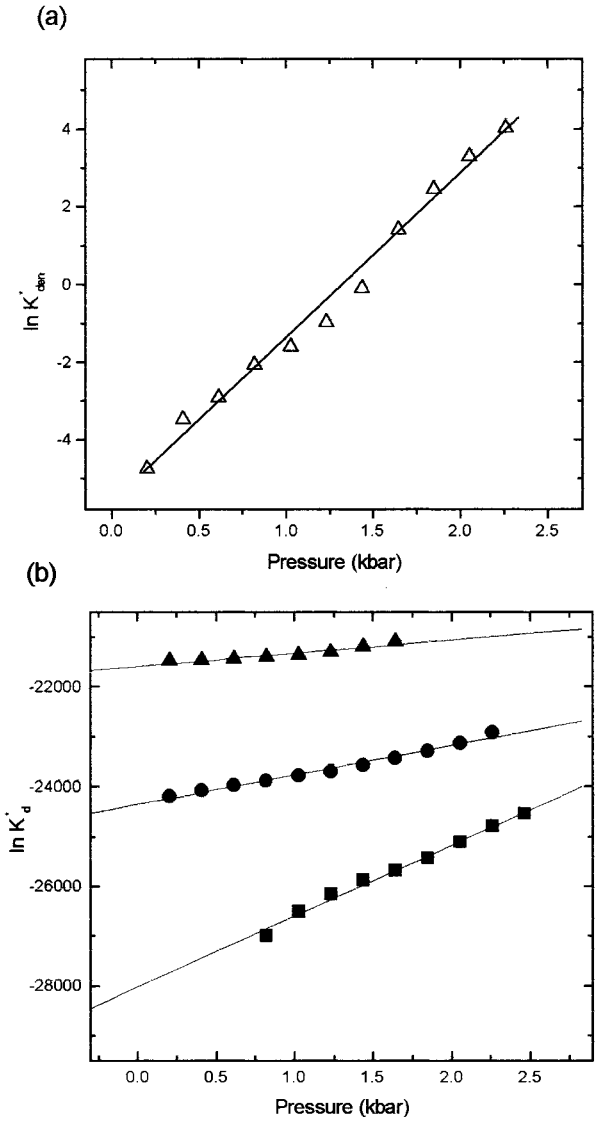

FIGURE 7: (a) Effect of pressure on the denaturation of TMV in the presence of 2.5 M urea based on center of mass (Figure 6b), curve corresponding to presence of 2.5 M urea. The calculation of ln $K^*_{den,p}$ versus pressure was based on the experiments of Figure 6b, center of mass data, and on eq 5. (b) Effect of pressure and urea on dissociation of TMV. The calculation of ln K^*_p versus pressure was based on the experiments of Figure 6, light-scattering data, and on eq 14. (■) Absence of urea and (●) 1.0, and (▲) 2.5 M urea. The standard deviations are smaller than the symbols used.

Table 1: Apparent Volume Change of Dissociation at Different Urea Concentrations Calculated from Eq 14 and Figure 7b

[urea] (M)	ln K^*_{atm}	ΔV^{0*}_{dis} (mL/mol subunit)
–	−28025	16.3
1.0	−24357	6.77
2.5	−21592	3.07

Pressure in the absence of urea induced a negligible denaturation process at different viral concentrations from 0.5 to 3.0 mg/mL, Figure 8a demonstrated through no shift of the center of mass of the emission fluorescence spectra. However, in the presence of 2.5 M urea, there was pressure-induced denaturation, Figure 8b. As expected for a first-order process, there was no concentration dependence on the pressure denaturation. To obtain the ΔV^{0*}_{den} based on eq 13,

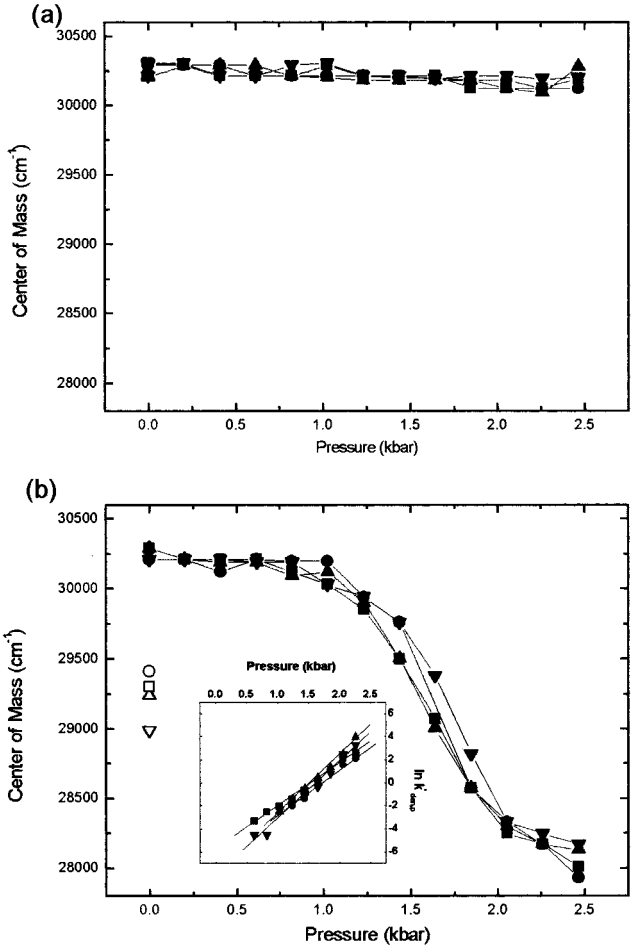

FIGURE 8: (a) Effect of pressure on center de mass of emission fluorescence spectra in different viral concentrations of TMV in the absence of urea. (b) Effect of TMV concentration on the pressure denaturation in the presence of 2.5 M urea, based on center of mass. (Open symbols) Return to atmospheric pressure. (Inset) ln K^*_{den} versus pressure based on eq 5. TMV concentration: 0.5 (▲), 1.0 (●), 2.0 (■), and 3.0 mg/mL (▼). The standard deviations are smaller than the symbols used.

Figure 8b, inset, shows the ln $K^*_{den,p}$ as a function of pressure for several TMV concentrations. The $p_{1/2,den}$ value was 1.60 ± 0.08 kbar, and ΔV^{0*}_{den} taken from the slopes of the curves gave a value of 101.0 ± 7.3 mL/mol subunit.

Decreasing the temperature down to −10 °C at 2.5 kbar did not induced the denaturation process in the presence of urea up to 1.0 M, Figure 9a. At higher urea concentrations, denaturation was observed upon decreasing the temperature. Figure 9b shows the van't Hoff plot at a pressure of 2.5 kbar in the presence of 1.75 M urea. The values for apparent enthalpy (ΔH^*_{den}) and entropy of denaturation ($T\Delta S^*_{den}$) were −11.1 and −10.2 kcal/mol subunit, respectively [$\Delta S^*_{den} = -36.3$ cal/(K mol of subunit)].

The effect of urea on TMV was also monitored by extrinsic fluorescence from bis-ANS (not shown). The interaction of virus with this probe was more intense at urea concentrations between 2.5 and 4.0 M.

Gel filtration analysis showed that 2.5 M urea incubation of TMV does not promote dissociation to molecular masses lower than 10^7 Da, which corresponds to the exclusion volume of column. TMV incubated at 2.5 kbar in the presence of 2.5 M urea presented elution corresponding to lower molecular masses (Figure 3b).

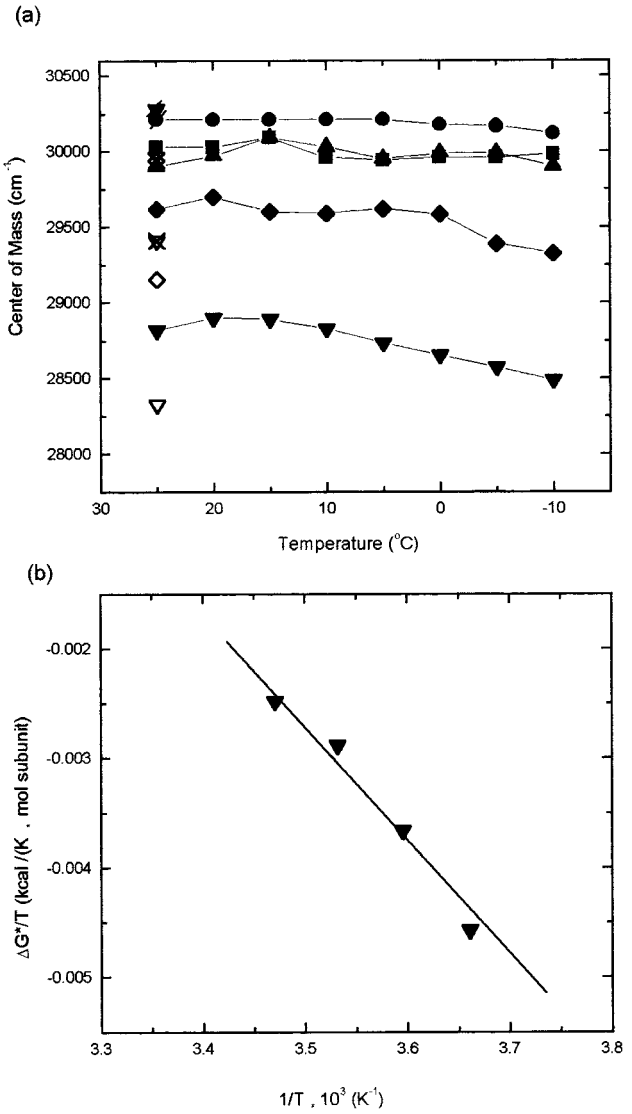

FIGURE 9: (a) Effect of temperature on center of mass of emission fluorescence spectra at 2.5 kbar and different urea concentrations. (●) Absence of urea and (■) 0.5, (▲) 1.0, (◆) 1.5, and (▼) 1.75 M urea. (Cut symbols) Samples at initial conditions at atmospheric pressure. (Open symbols) return to 25 °C at 2.5 kbar. (Crossed symbols) return to atmospheric pressure. TMV concentration: 1.0 mg/mL. (b) van't Hoff plot of TMV denaturation at 2.5 kbar and 1.75 M urea based on the experiment of panel a. The values of the degree of denaturation were calculated from eq 5, considering $\alpha_{den} = 1$, the center of mass in the presence of 6.0 M urea and under 2.5 kbar. The standard deviations are smaller than the symbols used.

The transmission electron microscopy showed that urea at 2.5 M promoted some TMV deaggregation at atmospheric pressure (Figure 10, panels a and b), and pressure incubation resulted in significantly more dissociated viral structures (Figure 10, panels c and d). The incubation in the presence of 6.0 M urea at 2.5 kbar promoted extreme viral dissociation based on structural images (see Figure 4d).

DISCUSSION

High Pressure on TMV: Little Dissociation. The incubation of TMV at pressures up to 2.5 kbar resulted in only 18% dissociation with negligible changes in the emission fluorescence spectra, indicating very little denaturation of subunits under these conditions. The extrinsic fluorescence studies with bis-ANS also demonstrated the absence of important changes in the tertiary structure of the coat protein, since there was no detectable difference between the fluorescence intensity of the probe at 2.5 kbar and atmospheric pressure. The transmission electron microscopy images of previously pressurized virus showed the same pattern as the native TMV.

The absence of RNA enormously decreases the stability of TMV, favoring pressure dissociation. TMV protein coat as double disks (10 mg of protein/mL) dissociates in the pressure range 0.1−0.5 kbar at 25.0 °C in pH 7.0 phosphate buffer (*6*). Helical rods (2.2 mg of protein/mL) did undergo dissociation in the range 0.1−0.6 kbar at 26.2 °C or 0.2−1.0 kbar at 29.7 °C in pH 6.5 phosphate buffer, with 50% dissociation, $(p_{1/2})_0 = 0.4$ kbar, and a volume change of dissociation (ΔV^0_{dis}) of about 86 mL/mol subunit. These data allow the estimation of the apparent free energy of stabilization given by viral RNA, based on the following equation (adapted from ref *16*):

$$\Delta\Delta G^*_{dis} = -[(p^*_{1/2})_R \, \Delta V^*_{dis,R}/n - (p_{1/2})_0 \Delta V^0_{dis}/n] \quad (15)$$

where $(p^*_{1/2})_R$ is the midpoint for pressure dissociation of the virion, that corresponds to about 7.0 kbar (extrapolation data, Figure 7b, lower curve), $\Delta V^*_R/n$ the apparent volume change of virion dissociation, calculated as −16.3 mL/mol subunit. The calculation of the apparent free energy of stabilization is −1.7 kcal/mol subunit, although there were some differences of pH and protein concentration between these experiments that, if corrected, should increase the apparent free energy of stabilization by RNA. The whole viral particle corresponds to an apparent free energy of stabilization of 3600 kcal/mol TMV.

The study of the icosahedral virus brome mosaic virus (BMV) (0.06 mg of virus/mL) presented a red shift in the emission fluorescence spectra of aromatic residues and significant changes in the interaction with bis-ANS at high pressure and on transmission electron microscopy during and after high-pressure incubation (*17*). Other icosahedral viruses, such as R17 bacteriophage (*18*) and cowpea mosaic virus (CPMV) (*16*) presented higher stability at high pressure. In the latter investigation, the important role of RNA on the stabilization of viral particle was shown by comparison of properties between purified coat protein and virus. The value of the free energy of stabilization was calculated as 0.988 kcal/mol subunit, which corresponds to a magnitude similar to TMV.

Namba et al. (*1*) have described the interaction of TMV protein coat and RNA based on X-ray fiber diffraction methods at 2.9 Å resolution. The phosphate groups are, in general, neutralized by arginine residues. Hydrophobic interactions occur between the bases and the protein, as well base-specific hydrogen bonds with the protein. The important energy of stabilization in TMV due to presence of RNA should be related to those interactions.

High Pressure at Low Temperature: Dissociation without Significant Denaturation. Decreasing the temperature down to −18 °C at 2.5 kbar promoted a high degree of dissociation with negligible denaturation (Figures 1, panels a and b and 9a). Only with a combination of pressure and low temperature it is possible to obtain dissociation, and in this case, there is a residual light-scattering peak corresponding to 28%

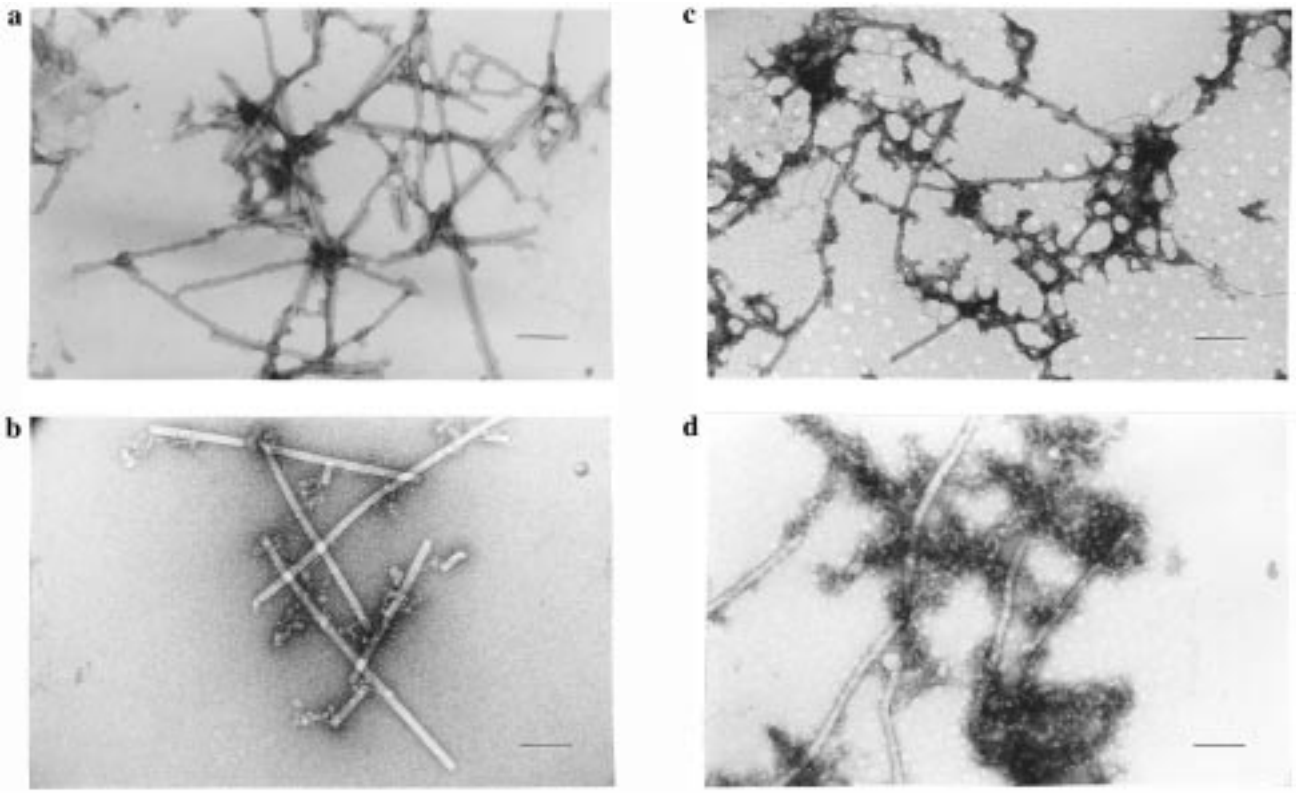

FIGURE 10: Transmission electron microscopy of TMV subjected to pressure in the presence of urea. 1.0 mg/mL TMV incubated with 2.5 M urea (a and b), and 2.5 M urea and subjected to 2.5 kbar for 1 h (c and d). Negative staining: 1% uranyl acetate. Other conditions as in Figure 1. The bar corresponds to 100 nm.

association. The transmission electron microscopy after incubation under pressure at low-temperature did not show any kind of particle or aggregate. Furthermore, the dissociation of TMV is related to large and negative values of apparent enthalpy and entropy (ΔH^*_{dis} and $T\Delta S^*_{dis}$), respectively, −8.23 and −14.5 kcal/mol subunit. As found for oligomeric proteins and CPMV (*14*, *19*), the measurement of these thermodynamic parameters for several proteins always showed that the contribution of the enthalpy changes to the free energy of dissociation is smaller than the changes in entropy ($T\Delta S_{dis}$) and are more often negative than positive, opposing association. The changes in entropy upon dissociation are large and negative and are responsible for the large preponderance of the aggregate over the isolated subunits (*19*). These values indicate that virion stability is an entropy-driven process. These results can be related to several kinds of interactions in the protein. Hydrophobic interactions due to exposure of apolar side chains to water promotes relevant decreasing of entropy due to local water organization. Privalov and Makhatadze (*20*) point out that the hydration of polar residues should also decrease the entropy. An alternative explanation of the entropy-driven condensation is the view that association of proteins is a result of the conversion of a considerable number of protein−water bonds into weaker protein−protein bonds (*19*).

High Pressure in the Presence of Urea: Dissociation and Denaturation. Urea promotes disassembly of TMV at 6.0 M and 0 °C (*21*, *22*). Blowers and Wilson (*23*) demonstrated that the viral dissociation occurs predominantly from the 5′-end of RNA.

Subdenaturing concentrations of urea promoted denaturation and dissociation induced by pressure. The shift of center of spectral mass and the light-scattering intensity decreases independently showed these processes (Figure 6).

The increasing of osmolarity by the presence of a solute should increase the stability of protein aggregates against dissociation (*24*, *25*). The dissociation and denaturation effect of pressure combined with subdenaturing concentration of urea overcomes the protection effect, demonstrating urea inclusion into the aggregate. The return to atmospheric pressure promoted partial reversibility of the pressure effect on dissociation and denaturation, especially at lower values of urea concentrations, as seen by the partial recovery of light-scattering intensity and center of mass values.

Figure 9a shows that, in the presence of urea at concentrations below the value that promotes denaturation at high pressure, there was no induction of denaturation by decreasing the temperature. However, at urea concentration of 1.75 M, decreasing the temperature induced additional denaturation, allowing the calculation of the apparent enthalpy and entropy of denaturation (ΔH^*_{den} and $T\Delta S^*_{den}$), respectively −11.1 and −10.2 kcal/mol subunit (Figure 9b). These results suggest that the process of denaturation by urea at high pressure is not driven by entropy. The limitation of these results lies in the values of the thermodynamic parameters which are apparent values since they were based on the steady-state condition. Although this approximation introduces some imprecision due to the fact that we are not dealing with a pure equilibrium, the results are undoubtedly correct in both sign and order of magnitude.

The dissociation and denaturation effect of pressure combined with subdenaturing concentration of urea and its reversibility were observed in other protein aggregates (*16*, *18*). Da Poian et al. (*14*) demonstrated the dissociation/

denaturation of CPMV by decreasing the temperature to −18 °C when the virus is incubated at 2.5 kbar in up to 1.0 M urea concentrations, following the red shift of the center of mass of fluorescence spectra. These results were taken as a dissociation process in the calculation of thermodynamic parameters, since dissociation and denaturation occurred concurrently. For TMV these processes could be separated.

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