We have analyzed the in vitro disassembly of the capsid of the minute virus of mice, and the stability of capsid chimeras carrying heterologous epitope insertions. Upon heating in a physiological buffer, empty capsids formed by 60 copies of protein VP2 underwent first a reversible conformational change with a small enthalpy change detected by fluorescence. This change was associated with, but not limited to, externalization of the VP2 N terminus. Irreversible capsid dissociation as detected by changes in fluorescence, hemagglutination activity, and electrophoretic mobility occurred at much higher temperatures. Differential scanning calorimetry in the same conditions indicated that the dissociation/denaturation transition involved a high enthalpy change and proceeded through one or more intermediates. In contrast, in the presence of 1.5 M guanidinium chloride, heat-induced disassembly fitted a two-state irreversible process. Both thermally and chemically induced dissociation/denaturation yielded a form that had lost a part of the tertiary structure, but still retained the native secondary structure. Data from chemical dissociation indicates this form may correspond to a molten globule-like monomeric state of the capsid protein. All five antigenic peptide insertions attempted in exposed loops, despite being perhaps among the least disruptive, led to defects in folding/assembly of the capsid and, in most cases, to reduced capsid stability against thermal dissociation. The results with one of the simplest viral capsids reveal a complex pathway for disassembly, and a reduction in capsid assembly and stability upon insertion of peptides, even within the most exposed capsid loops.

The study of the folding, association, and disassembly of large multimeric proteins is complicated by their size, the general irreversibility of the reactions involved, and the frequent occurrence of off-pathway intermediates. However, the significant advances already made hold promise for a detailed understanding of these processes (1). Spherical virus capsids are large, multimeric proteins (2–5) and constitute attractive models for the study of the association, stability, and disassembly of very large protein complexes (for reviews see Refs. 4 and 6–13). In addition, viral capsids are highly dynamic entities and have evolved unique structural solutions in response to the diverse, sometimes conflicting functions they must perform during the viral life cycle (4, 7, 11, 14–16). They provide good opportunities to understand finely tuned structure-function relationships in proteins and to develop new antiviral approaches based on the inhibition of assembly or uncoating (15, 17, 18).

The icosahedral T = 1 capsids of parvoviruses (19–24) are formed by 60 protein subunits contributed by three nonidentical polypeptide chains that show, however, identical -fold and core sequence. VP2 is the major capsid protein and can self-assemble into empty (DNA-free) capsids (viral-like particles or VLPs).1 VP1, a minor component of natural capsids, includes the VP2 sequence plus an N-terminal extension. VP3 is produced only in assembled virions by the proteolytic removal of a short N-terminal segment in a fraction of VP2 subunits (25). The structure, stability, and other physical and antigenic properties of empty capsids of parvoviruses containing only VP2 are very similar to those of native capsids containing also VP1 (26).2 Such VLPs thus constitute very simple models to study the mechanisms of assembly, stability, and disassembly of parvoviruses and of large macromolecular assemblies in general.

In vitro, assembly of autonomous parvoviruses may proceed through a trimeric intermediate (20, 27, 28) that is transported to the cell nucleus (27, 28). Uncoating may involve acidification at the endosomes (29, 30), but the disassembly pathway is also unclear. In vivo, assembly and uncoating of parvoviruses, or of many other viruses, are incompletely understood.

Chimeric parvoviral VLPs and other viral capsids carrying heterologous epitopes derived from diverse pathogens hold considerable promise to develop new vaccines (reviewed in Refs.

The abbreviations used are: VLP, viral-like particle; CPV, canine parvovirus; DSC, differential scanning calorimetry; GdnHCl, guanidinium hydrochloride; MVM, minute virus of mice; PBS, phosphate-buffered saline; Tm is the half-transition temperature except for DSC analyses where it denotes the temperature of the maximum in the heat capacity function and may be equal or higher than the half-transition temperature, depending on the denaturation mechanism.

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2 N. Valle and J. M. Almendral, unpublished observations.
Stability and Disassembly of a Parvovirus Capsid

33–37). Chimeric viruses, and also chimeric VLPs from human parvovirus B19 (38, 39), porcine parvovirus (40–42), or CPV (43), (i) are recognized by neutralizing antibodies against the donor pathogen, (ii) elicit immune responses specific of the epitopes inserted, and/or (iii) induce protection in animals against infection with the donor virus. Parvovirus capsids present large loops where insertions can be made, and are extremely stable against heat and other denaturing agents. However, large deletions in three exposed loops on the surface of VLPs from CPV seriously impaired expression and assembly (44). Insertion in these loops of a 10- to 13-mer amino acid peptide, avoiding deletion of any capsid residue, allowed the recovery of chimeric VLPs but they were obtained, at least in some cases, in reduced amounts (43). Likewise, the capsids of the adeno-associated parvovirus were obtained in reduced yields and showed functional constraints when short peptides were inserted with the purpose to alter tissue tropism (45, 46). Reduced titers or fitness of virus chimeras, or low production of chimeric particles, have been repeatedly noted (e.g. Refs. 43, 47, and 48). Because of fundamental thermodynamic considerations (49, 50) and the complex oligomeric nature of viral capsids, even the least disruptive peptide insertions could have negative effects on assembly and/or decrease capsid stability against dissociation. However, to our knowledge the effect on the thermostability of any viral capsid of peptide insertions has remained essentially untested to date.

We have chosen the empty, VP2-only capsid of the parvovirus MVM as a model of icosahedral virus capsids and other macromolecular assemblies. Biochemical and biophysical techniques were used here to provide further insights on the in vitro stability and disassembly of one of the simplest viral capsids, and to analyze the effects on capsid stability against dissociation of rationally designed heterologous insertions in surface loops.

EXPERIMENTAL PROCEDURES

Recombinant Plasmids—Plasmid pSVTK-VP1/VP2 included the VP1/VP2 gene of MVMp and was derived from a molecular clone of the MVMp genome (51). The VP2-coding segment was amplified, using PCR and oligonucleotides with flanking BamHI and XhoI sites. The purified DNA segment and vector pFastBac1 (Invitrogen) were digested with BamHI and XhoI, re-purified by agarose gel electrophoresis, and ligated using T4 DNA ligase. The recombinant plasmid (pB1-VP2) was obtained from transformed Escherichia coli DH5α, and the inserted VP2 gene and flanking segments were sequenced using an automated method. The Bac-to-Bac Baculovirus Expression System (Invitrogen) was then used to construct a baculovirus shuttle vector (bacmid) containing the VP2 gene, using pB1-VP2 as the donor plasmid (52). The manufacturer instructions were followed with minor modifications. The recombinant bacmid, BM-VP2, was isolated from a positive bacterial colony, and purified following a modified alkaline lysis procedure. The VP2 segment in BM-VP2 was also sequenced.

Site-directed Mutagenesis—Point mutations were introduced in pB1-VP2 and pSVTK-VP1/VP2 by the inverse PCR method using the QuikChange site-directed mutagenesis system (Stratagene). Insertions were introduced by either of two methods: (i) a unique restriction site was created in pB1-VP2 at the chosen insertion site on VP2. Partially overlapping oligonucleotides were used to obtain by PCR a DNA segment containing the sequence to be inserted, flanked at both ends by the appropriate restriction site. This DNA segment was purified, digested with the corresponding restriction endonuclease, and ligated with an excess of VP2 previously digested with the same nuclease, dephosphorylated and purified (53). (ii) Alternatively, mutagenesis was by inverse PCR on pB1-VP2 or pSVTK-VP1/VP2 using the QuikChange system, but following a modified procedure (54) optimized for long oligonucleotides. Each mutation introduced was confirmed by sequencing.

Expression of VLPs in Insect Cells—About 3 × 10⁶ H-5 cells from semiconfluent monolayers were resuspended in 2 ml of TC-100 medium supplemented with 5% fetal calf serum, and adsorbed in a single well from a 6-well tissue culture plate at 27 °C for 1 h. The medium was removed, the cells were washed with TC-100, and 1 ml of transfection mixture (20 µl of BM-VP2 DNA, 6 µl of Collefect reagent (Invitrogen) in 174 µl of TC-100, incubated at room temperature for 30 min, and diluted with 800 µl of TC-100) was added. After further incubation at 27 °C for 5 h, the transfection mixture was removed and 2 ml of TC-100 + 5% fetal calf serum were added. The cells were incubated at 27 °C until nearly complete cytopathic effect (about 6 days). The cell suspension was collected and microcentrifuged at 4 °C, and the supernatant was stored at −70 °C as a source of recombinant baculovirus. Titration of baculovirus was carried out as described (55); the titer obtained was around 1 × 10⁶ plaque-forming units/ml. The cell pellet was resuspended in phosphate-buffered saline (PBS) and analyzed by SDS-PAGE for VP2 expression. The recombinant baculovirus obtained was used to infect 70% confluent H-5 monolayers at a multiplicity of infection of 0.1–0.2, and the cells were harvested at 4 °C for 4 days, harvested, and centrifuged at 4 °C. The supernatant was collected and stored at −70 °C as a source of recombinant baculovirus (titer around 2–4 × 10⁷ plaque-forming units/ml). The cell pellet was resuspended in the same volume of PBS and centrifuged again. The washed pellet was then thoroughly resuspended in lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 0.2% Triton X-100, 0.2 mM EDTA), and frozen at −70 °C as a source of MVM capsids. For large scale capsid production, modifications included the use of 80% confluent H-5 cells, a multiplicity of infection around 1, and harvesting after complete cytopathic effect (3 days post-infection, a time when VP2 expression reached a maximum).

Purification and Sedimentation Analysis of VLPs—The procedure was derived from that described in Ref. 26. Except where indicated, all steps were carried out at 0–4 °C. Frozen infected cell extracts were thawed, sonicated, and clarified by centrifugation (10,000 rpm, 15 min). The supernatant was loaded in centrifuge tubes containing 1.6-mL density cushions (20% sucrose in PBS) and centrifuged at 10 °C in a SW40 rotor (Beckman) (16,000 rpm, 15 h). Each pellet was thoroughly resuspended in 0.5 ml of PBS and microcentrifuged. For some preparations, the capsid was precipitated with 20% ammonium sulfate (53) instead of centrifuged through a sucrose cushion. In either case, the concentrated capsid solution was loaded on a 12-mL density gradient (10–40% sucrose in PBS) and centrifuged at 5 °C in a SW40 rotor (Beckman) (16,000 rpm, 5.5 h). Fractions were collected and analyzed by SDS-PAGE. Those that contained capsids were pooled, extensively dialyzed against PBS and either kept at 4 °C or stored at −70 °C. For further purification, the capsids were centrifuged in a cesium chloride gradient in PBS (average density 1.38 g cm⁻³) for 29.5 h at 10 °C and 50,000 rpm in a TFP77.13 rotor (Kontron). Capsid-containing fractions (density 1.32 g cm⁻³) were collected and microcentrifuged at 80,000 rpm for 4 days, harvested, and centrifuged at 4 °C. The supernatant was collected and microcentrifuged at 4 °C as a source of recombinant baculovirus (titer around 2–4 × 10⁷ plaque-forming units/ml). Each pellet was resuspended in phosphate-buffered saline (PBS) and centrifuged again. The washed pellet was then thoroughly resuspended in lysis buffer (PBS) and centrifuged at 4 °C, 35,000 rpm, 30 min, and the supernatant was collected and stored at −70 °C as a source of recombinant baculovirus. Titration of baculovirus was carried out as described (55); the titer obtained was around 1 × 10⁶ plaque-forming units/ml. The cell pellet was resuspended in the same volume of PBS and centrifuged again. The washed pellet was then thoroughly resuspended in lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 0.2% Triton X-100, 0.2 mM EDTA), and frozen at −70 °C as a source of MVM capsids. For large scale capsid production, modifications included the use of 80% confluent H-5 cells, a multiplicity of infection around 1, and harvesting after complete cytopathic effect (3 days post-infection, a time when VP2 expression reached a maximum).

Circular Dichroism (CD) Spectroscopy—CD measurements were carried out using a Jasco-810 spectropolarimeter equipped with a computer-operated Peltier temperature control unit. Purified VLP preparations were concentrated by ultrafiltration in Centricon 100 units (Amicon). Purity was assessed by SDS-PAGE and UV spectrophotometry, and the capsid (monomer) concentration was determined using an extinction coefficient of 280 nm ε₂₈₀ = 114,160 M⁻¹ cm⁻¹ (56).

Fluorescence Spectroscopy—A Varian Cary Eclipse luminescence spectrophotometer equipped with a computer-operated Peltier temperature control unit was used. Purified VLP solutions at a defined protein concentration of 5 µM in a 1-mm path length cell were used. The recorded far-UV spectra were the average of 3–5 scans obtained at a rate of 50 nm/min, a response time of 2 s, and a bandwidth of 1 nm. The temperature was kept constant at 25 °C. Thermal denaturation experiments were carried out using the temperature scan mode and measuring the ellipticity in the far-UV at 215 nm. A temperature scan rate of 20 °C/h, a response time of 2 s, and a bandwidth of 1 nm were used. The far-UV data were fitted to a two-state model by monitoring the temperature dependence in the CD transition with a time constant of 15 ms.

Fluorescence polarization—Polarization values were obtained using a probe inside the cell.
cuvette were determined by using a submerged probe, and the registered (block) temperature corrected accordingly. Equilibrium data corresponding to the reversible transition observed were fitted to a simple cooperative unimolecular process using the program Kaleidograph and the equation,

$$I = (I_a + m_aT) - [(I_b + m_bT) - (I_a + m_aT)] \exp(- \exp(E_{\text{app}}/T) - T_m)]$$

(Eq. 2)

which was derived following the procedure described by Sanchez-Ruiz (57) for the temperature dependence of heat capacity. $E_{\text{app}}$ is the apparent activation energy for the rate-limiting step of the reaction.

Kinetic measurements were performed at temperatures between 60 and 80 °C, and data were collected every second. The experimental kinetic data were fitted to a single exponential, first-order decay curve using the equation,

$$I = I_0 \exp(-kt) + I_1$$

(Eq. 3)

where $I$ is the fluorescence intensity at time $t$, $I_0$ is the fluorescence intensity at $t = 0$, $h$ is the dissociation rate constant, and $I_1$ is the fluorescence intensity at infinite time.

**Differential Scanning Calorimetry (DSC)—Measurements were performed using a Microcal MCS instrument. Samples were in PBS with or without 1.5 M GdmHCl and were maintained under an extra constant pressure of 2 atm to prevent degassing during the scan. The MCS Observer and Origin software packages were used for data acquisition and analysis.**

The excess heat capacity functions were obtained after baseline subtraction of the buffer-buffer baseline. Protein samples for DSC were extensively dialyzed against the appropriate buffer, and the baseline correction was made using a Microcal MCS instrument. Samples were in PBS with or without 1.5 M GdmHCl and were maintained under an extra constant pressure of 2 atm to prevent degassing during the scan. The MCS Observer and Origin software packages were used for data acquisition and analysis.

**Heat-induced Conformational Transitions in VP2-only Capsids—Biochemical analyses had shown that virions and empty capsids of parvoviruses dissociate at about 70–80 °C in physiological buffers (26, 31, 32). We have subjected purified VLPs of MVM to linear thermal gradients from 25 to about 90 °C, and carried out agarose gel electrophoresis and hemagglutination activity assays, intrinsic Trp fluorescence and far-UV CD spectroscopy, and DSC analyses to reveal changes in quaternary, tertiary, and secondary structure.**

The tryptophan fluorescence emission spectrum of the VP2-only MVM capsid showed a maximum at about 336 nm (Fig. 1A), consistent with the buried positions of essentially all of the 15 Trp (per monomer) in the capsid structure (22, 23). Changes in the Trp fluorescence intensity at 330 nm upon heating of capsid solutions in PBS revealed two well separated cooperative transitions, superimposed with the expected linear decrease in intensity due to thermal quenching (Fig. 1, B and C). Such transitions indicated changes in Trp exposure to solvent, and thus involve quaternary and/or tertiary structural alterations. The $T_m$ value for each transition was independent of the heating rate.

The first transition (Fig. 1B) occurred without capsid dissociation, and was marked by a small but very reproducible decrease in Trp fluorescence with a $T_m = 64.3 \pm 0.9$ °C. Heating to 53 °C (a temperature at which the first transition had been completed) and cooling showed this transition to be fully reversible, thus allowing equilibrium analysis. The $T_m$ value obtained was independent of protein concentration, and the transition could be well fitted to a simple unimolecular two-state process that yielded an approximate enthalpy change $\Delta H^\circ = 108$ kcal per mol of cooperative unit. We carried out thermal gradient experiments in the presence of trypsin to determine whether the conformational change observed by fluorescence and gel electrophoresis of the VP2 N termini detected in VLPs heated to high temperature (26) could be associated. Cleavage of VP2 started at about 42 °C and reached a maximum of about 25% of the VP2 molecules present (Fig. 2). The externalization of the N terminus upon heating was found to be a reversible process (not shown) with an approximate $T_m = 44.4 \pm 1.3$ °C (Fig. 2), which corresponded, within error, with that of the first (reversible) transition observed by fluorescence.

The second transition observed by fluorimetry (Fig. 1C) was marked by a more substantial decrease in tryptophan fluorescence intensity and spectral red shift and yielded a transition temperature $T_m = 74.9 \pm 0.6$ °C. It was irreversible, and it did not depend on the capsid concentration used, as expected if cooperative intraparticle interactions, and not interparticle interactions, were involved. Quantitative analyses of particle size
by gel electrophoresis, and of capsid integrity in hemagglutination assays directly showed that the capsid started to dissociate above 70°C, and that the dissociation process could be fitted to a simple cooperative transition with a \( T_m \) of 74.8 ± 0.5° C or 74.4 ± 0.8°C, respectively (Fig. 3), values that corresponded with that of the second conformational transition detected by fluorescence (Fig. 3 and Table I).

Heat-induced dissociation/denaturation of the capsid in PBS led to visible protein precipitation, which precluded spectroscopic analyses of any further transition after dissociation. Thus, the above experiments were repeated in the presence of 1.5 M GdmHCl in PBS. This denaturant concentration was not enough to dissociate the MVM capsid, as shown by gel filtration chromatography and fluorescence analyses, but avoided precipitation upon capsid dissociation. In these conditions, the low-temperature conformational transition was not observed. Trypsin sensitivity assays showed that, in the presence of this concentration of GdmHCl, about 25% of the VP2 N termini were already externalized at low temperatures, and this percentage did not change upon heating at 52°C (not shown). In addition, both heating to about 50°C or the addition of 1.5 M GdmHCl at 25°C caused a very small red shift (about 1 nm) in the wavelength of maximum fluorescence emission of the tryptophans, which appeared to be reversed when the samples were brought back to the original conditions. Furthermore, GdmHCl dissociation was again irreversible and independent of protein concentration, and moderately dependent on the

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**Fig. 1.** Thermal dissociation/denaturation of the MVM capsid probed by fluorescence. A, tryptophan fluorescence emission spectrum of the MVM capsid in PBS before (solid line) and after heating to 85°C (dotted line). B–D, fluorescence intensity at 330 nm of the MVM capsid in PBS (B and C) or in PBS with 1.5 M GdmHCl (D) as a function of temperature. For reasons of scale and clarity, the complete curve obtained in the same experiment in PBS has been split into two parts corresponding to low (B) and high (C) temperature ranges. **a.u.**, arbitrary units.

**Fig. 2.** Heat-induced externalization of the N terminus of VP2 in the capsid. The fraction of VP2 externalized at different temperatures, as determined by SDS-PAGE and densitometry, is indicated (circles). The data have been fitted to a sigmoidal transition.

**Fig. 3.** Apparent fraction of dissociated/denatured capsid versus temperature. The data obtained by intrinsic Trp fluorescence (second transition, Fig. 1C) (large circles), capsid hemagglutination activity (triangles), agarose gel electrophoresis (inverted triangles), and DSC (small circles; only one of every five data points is shown) were normalized to obtain the apparent fraction of dissociated/denatured capsid at each temperature. Fitting to a sigmoidal transition of the values obtained by fluorescence (solid line), hemagglutination (dotted line), or agarose electrophoresis (dashed line) yielded nearly coincident curves.
heating rate. Both in the absence or presence of 1.5 M GdmHCl, at temperatures close to the \( T_m \), the decrease in fluorescence intensity over several minutes could be adequately fitted to a single exponential decay curve, consistent with a single major, first-order capsid dissociation step (data not shown). The dissociation rate constant obtained at 61.5 °C in the presence of 1.5 M GdmHCl was 0.13 min^{-1}.

Highly purified VLPs were subjected to differential scanning calorimetry analyses either in the absence or presence of 1.5 M GdmHCl. Both thermograms (Fig. 4) showed only one asymmetric \( \Delta C_p \) peak with \( T_m \) values of 77.6 or 62.1 °C, respectively (Table I). The absence of any transition at temperatures around 46 °C indicated that the cooperative unit involved in the first transition observed by fluorescence of capsids in PBS is probably large, so that the monomer contribution to the enthalpy change is too low to allow detection by DSC at the available capsid concentrations.

Dissociation/denaturation of the MVM capsid in PBS proceeded with a total enthalpy change \( \Delta H^\text{app} = 486 \pm 20 \text{ kcal/(mol of monomer)} \), and the \( T_m \) did not vary upon increasing the heating rate from 30 to 45 °C/h. Protein precipitation started below the \( T_m \) obtained by DSC, which suggests that the irreversibly denatured state became significantly populated within the transition interval, and that the dissociation/denaturation process did not occur under quasi-equilibrium conditions, but was under kinetic control. The apparent independence of the scan rate could be explained if the activation energy \( E_{\text{app}} \) of the first-order rate-limiting step is high. In such a case, variations in \( T_m \) would be observed only between scans run at widely different rates (57, 62, 63). The profile of the apparent fraction of dissociated/denatured capsid versus temperature, as calculated from the DSC curve, differed from those obtained with biochemical probes of capsid dissociation (Fig. 3), and indicated that the major variation in enthalpy occurred after capsid disruption. This suggests that capsid disassembly and denaturation may proceed through formation of intermediate(s). Analysis of the thermogram also showed that above 75 °C the experimental curve can be described by a two-state irreversible process, but deviates from this behavior at lower temperatures (Fig. 4A).

In contrast, in the presence of GdmHCl thermal dissociation/denaturation could be described in terms of a single two-state irreversible process with first-order kinetics (Fig. 4B; Table I). According to this model, a rate constant of 0.127 min^{-1} at 61.5 °C was calculated from the DSC data, in good agreement with the analysis of fluorescence data under the same conditions (see above).

Thermal denaturation of VLPs was also characterized by CD in the presence of 1.5 M GdmHCl, where no protein precipitation occurred (Fig. 5). The far-UV spectrum obtained in the presence of 1.5 M GdmHCl was similar to that obtained in its absence. The small quantitative difference (about 10%) amounted to less than 2 millidegrees in the raw ellipticity.

![Fig. 4. DSC thermograms of the MVM capsid. A, heat capacity curve (scan rate 30 °C/h) of the MVM capsid (4.8 μM monomer concentration) in PBS. The dashed line indicates the expected behavior for a single irreversible two-state model with first-order kinetics (\( T_m = 77.6 °C; \Delta H^\text{app} = 143 \text{ kcal/(mol of monomer)} \); \( E_{\text{app}} = 233 \text{ kcal/(mol of monomer)} \)). B, heat capacity curve (scan rate 21.5 °C/h) of the MVM capsid (4.6 μM monomer concentration) in 1.5 M GdmHCl; the continuous line is the theoretical fitting to an irreversible two-state model (Table I).](image-url)
would be needed. In addition, the screening of the charges on the protein surface by guanidinium and/or chloride ions could contribute to a small increase in the absolute ellipticity values (64). The far-UV CD spectrum remained essentially unchanged at any temperature tested, even at 95 °C (Fig. 5B). This suggested that thermal dissociation/denaturation of the MVM capsid leads to a stable form that preserves the native secondary structure (see below and the “Discussion”).

GdmHCl-induced Conformational Transitions in VP2-only Capsids—Chemical dissociation/denaturation of the capsid at a moderate temperature (25 °C) was also carried out, using GdmHCl as denaturant. The presence of low concentrations of GdmHCl were apparently enough to trigger externalization of the VP2 N terminus (see above). In addition, tryptophan fluorescence analysis (Fig. 6) revealed a single irreversible, sigmoidal transition with a D50% (the denaturant concentration at which the process was half-completed) of 3.4 M (Fig. 6B) and a reduction in the fluorescence quantum yield as well as a red shift of the maximum intensity to 353 nm (Fig. 6A). Again, the transition did not depend on protein concentration, as expected from an irreversible process that does not involve protein aggregation. Far-UV CD analysis showed that the native secondary structure was essentially fully preserved at or below 4.5 M GdmHCl (Fig. 6B). The non-coincident far-UV CD and fluorescence titration curves indicate, as observed for the thermally induced dissociation process, the presence of a stable disassembly/denaturation product that maintains the native secondary structure. Gel filtration chromatography of MVM capsid preparations at 4 M GdmHCl, where this form appears to be highly populated, revealed no aggregate, and indicated an apparent molecular weight of about 80,000–90,000, which was 30–40% higher than expected for a compact VP2 monomer. Even after removal of the GdmHCl, the product of capsid dissociation/denaturation was found monomeric by sucrose gradient centrifugation, and no significant amounts of higher molecular weight species were present (data not shown).

Effect of Insertion of Heterologous Peptide Sequences in Surface Loops on the Thermostability of VLPs—The Tm of the dissociation step detected by fluorescence or by quantitating hemagglutination activity proved to be a very precise parameter to compare the stability of mutant parvoviral VLPs against heat-induced dissociation (see above). We have used this parameter to determine the relative thermostability of engineered MVM particles with heterologous peptide segments inserted in capsid loops.

Two viral antigenic peptides that contain well characterized continuous B-cell epitopes were selected as models: the B-C loop of VP1 of poliovirus type 3 (65), previously used to test the immunogenicity of CPV chimeras (43), and the G-H loop of VP1 of FMDV type C (14) (Table II). The three-dimensional structure of the MVM capsid (22, 23) was inspected for sites of insertion that could have the least disruptive effects on capsid stability. The positions chosen were located close to the tips of some loops highly exposed to solvent on the capsid surface, and involved in few or no interactions with residues in neighboring loops, especially those in other subunits. Consideration was also given to the best preservation of intraloop interactions by generally avoiding the deletion of VP2 residues, and by favoring insertion sites not contiguous to residues involved in hydrogen bonds or multiple intraloop van der Waals contacts. In addition, sites that could allow little spacing between the 60 identical peptides to be inserted (e.g. the loops closest to the 5-
and 3-fold symmetry axes) were not considered. The four specific sites finally selected for insertion are listed in Table II and shown in Fig. 7. Three capsids with point mutations were also obtained. All of them involved a chemically conservative substitution (from Thr to Ser) that deleted just a methyl group in an exposed side chain oriented to solvent, not involved in any intracapsid interaction, and each mutation was located very close to a site chosen for epitope insertion (Table II and Fig. 7).

Two of the capsids with point mutations (T231S and T515S) accumulated in insect cells and self-assembled into VLPs to the same high levels obtained with the parental capsid, as expected. In contrast, VP2 of one point mutant (T90S) and all four chimeras yielded relatively low amounts of capsid. The nonmutated protein, T90S and three chimeras were also expressed in mammalian cells as empty capsids containing both VP1 and VP2, and analyzed by immunofluorescence (Fig. 8) using a polyclonal antibody that recognizes the capsid protein subunits, and a monoclonal antibody specific for assembled capsids (VP2, and analyzed by immunofluorescence (Fig. 8) using a polyclonal antibody that recognizes the capsid protein subunits, and a monoclonal antibody specific for assembled capsids.

TABLE II

Peptide insertions and point mutations in MVM capsids

| Mutant | Amino acid sequence |
|--------|---------------------|
| **Insertions** | |
| 93PV   | 230TDTSNPASTTNKDVKGN |
| 229PV  | 230QEGTDNPSATTNKDVH |
| 390PV  | 230QGADNPSATTNKDGVA |
| 513PV  | 230KNASHGDNPSATTNKDGVPAP |
| 229FMDV | 230QGAYTASARGDLAHITTTG |
| **Point mutations** | |
| T90S   | 230THNTSNGS |
| T231S  | 230NQEGSVEH |
| T515S  | 230PNGALSRLV |

The two single mutant VLPs that were normally expressed in insect cells and the four chimeras, despite their low yields, could be purified from these cells. All of these capsids had the expected size as revealed by ultracentrifugation in sucrose gradients, and were exclusively composed of intact VP2 with the expected molecular weight in SDS-PAGE. These capsids were subjected to thermal stability assays, with the dissociation step being followed by quantitating the residual hemagglutination activity and changes in Trp fluorescence, and the corresponding $T_m$ of dissociation was determined (Table III) as described above for the nonmutated capsid. The two point mutants and one of the chimeras (513PV) yielded a $T_m$ that was indistinguishable, within error, from that of the parental capsid. In contrast, the stabilities of the three other chimeras were substantially reduced, as reflected in a $T_m$ that was about 3 °C (chimera 229PV), 6 °C (chimera 390PV), or 15 °C (chimera 93PV) lower that of the nonmutated capsid (Table III). For comparison, acidification of the nonmutated capsid to a pH as low as 2.7 reduced the $T_m$ by 16 °C. The insertion of the FMDV peptide (chimera 229FMDV) instead of the poliovirus peptide (chimera 229PV) yielded essentially the same result (not shown). To summarize these data, one of three point mutations and all five insertions attempted, despite being made at positions that are presumably among the least disruptive for stability of this robust viral capsid, led to defects in capsid subunit accumulation and capsid self-assembly in insect and mammalian cells. Moreover, all of the assembled chimeras but one showed a clearly reduced stability against dissociation by heat. These results suggest a participation of even the most exposed, loosely connected loops of VP2 on the assembly of a parvovirus capsid and its stability against dissociation.

**DISCUSSION**

Some viruses meet the conflicting biological requirements of extracellular stability versus intracellular lability by evolving a metastable capsid, whose conformation and stability are modulated during the life cycle. Similar conformational changes in vitro may be induced by heat (66) or other denaturants. The results described here, taken together, suggest that in vitro disassembly of the VP2-only capsid of MVM, one of the structurally simplest viral protein shells, is a complex process that may occur as follows. Prior to the dissociation step the capsid undergoes a reversible conformational rearrangement. This change occurs at moderate temperatures ($T_m = 46$ °C in PBS) and does not require VP1 or the presence of the viral DNA. It does not produce a detectable variation in particle size, hemagglutination activity, heat capacity, or protein secondary structure, but it may be detected by a slight change in the exposure of some buried tryptophans to solvent. This conformational rearrangement is associated with externalization of...
the N terminus of about 20–25% of the capsid subunits. The effect of moderate heating could be also achieved by addition of 1.5 M GdmHCl, which led to externalization of about 25% of the VP2 N termini. Crystallographic evidence indicates that externalization of the N terminus of the capsid proteins may occur through the capsid 5-fold axes (19, 23). Interestingly, no Trp is located spatially close to the 5-fold axes in the MVM capsid, so the observed conformational change cannot be just a local event, but may also involve a more global rearrangement of the capsid structure. However, this change may be of a subtle nature, because it would involve an enthalpy variation of only 1.8 kcal per mol of monomeric subunit (if the entire capsid is assumed to be the cooperative unit, which is consistent with this transition being undetected by DSC). Our analysis of mutant capsids with single alanine substitutions has revealed that some mutations did not affect this transition, whereas others prevented this conformational rearrangement from occurring. Preliminary evidence indicates that externalization of the N terminus and/or the associated change of capsid conformation are essential for virus infectivity. This latter event could be necessary to facilitate uncoating in vivo.

The dissociation step occurs at much higher temperatures (\( T_m \) = 75 °C in PBS). The DSC results suggest that this step may proceed first with dissociation into an intermediate that exposes some Trp to solvent (as seen by fluorescence). The intermediate would subsequently further dissociate (if not already monomeric) and/or denature, with a much larger enthalpy change. Trimeric of the capsid protein may constitute intermediates of parvovirus assembly (see the Introduction), and in the native capsid the number of intertrimer interactions is much lower than that of intratrimers or intramonomer interactions (20). Thus, one possibility consistent with the results obtained, is that the disassembly intermediate proposed could correspond to a trimeric, perhaps partly denatured, form of the capsid protein. Heat-induced denaturation in 1.5 M GdmHCl conformed to an irreversible first-order, two-state process, suggesting that addition of this low concentration of a chaotropic agent may be enough to destabilize the intermediate, whose detection may thus critically depend on the conditions used. Although a certain contribution from the large aggregates formed at high temperature in PBS cannot be excluded, the strong reduction in \( T_m \) and \( \Delta H^\text{m} \) values observed upon addition of 1.5 M GdmHCl would reflect variations in inter- and/or intra-subunit interactions in the capsid. The product obtained by both thermal or chemical dissociation/denaturation lacked a substantial part of the tertiary structure but preserved the full secondary structure of the complete capsid. The results of chemical dissociation/denaturation experiments in particular suggest that this product is not a fully denatured VP2 molecule, but may resemble a monomeric molten globule-like form of VP2. In the capsid structure, each VP2 subunit displays a relatively small \( \beta \)-sandwich core and many extremely long loops that are intertwined with those of neighboring subunits, especially within trimers (20). It is conceivable that, upon dissociation of the capsid, and of the proposed trimeric intermediate (if present), many of those loops would partially unfold, whereas preserving the secondary structure in the relatively small \( \beta \)-sandwich core of VP2. In vitro disassembly of a very simple virus capsid appears more complex than anticipated. Further studies are required to integrate these and previous observations (26, 31, 32) for a more complete understanding of disassembly mechanisms and of parvovirus uncoating.

The present characterization of the disassembly process validated the use of the \( T_m \) value obtained for the dissociation step in controlled thermal gradient experiments, followed either by fluorescence or hemaggulination activity, as a reliable indicator of the kinetic stability of mutant MVM capsids. Several groups have already investigated the antigenicity and immunogenicity of chimeric virions and VLPs (33–42, 47, 48). One of the aims of the present work has been to complement those studies by focusing not on the immunogenicity, but on the stability of the chimeric parvoviral particles instead. Given the structural similarity among the capsids of autonomous parvo-

### Table III

**Assembly and dissociation of MVM chimeras and variants with point mutations in surface-exposed loops**

| Capsid     | Yield in insect cells | Capsid size | \( T_m^\text{a} \) (fluorescence) | \( T_m^\text{a} \) (hemagglutination) |
|------------|-----------------------|-------------|-----------------------------------|--------------------------------------|
| Parental   | Normal                | Normal      | 74.9 °C ± 0.6                      | 74.4 °C ± 0.8                       |
| Chimera 93PV | Low                   | Normal      | 59.3 °C ± 0.1                      | 60.6 °C ± 0.1                       |
| Chimera 229PV | Low                  | Normal      | 73.0 °C ± 0.3                      | 70.7 °C ± 0.4                       |
| Chimera 390PV | Low                  | Normal      | ND\(^b\)                           | 68.2 °C ± 0.7                       |
| Chimera 513PV | Low                  | Normal      | 74.8 °C ± 0.5                      | 74.7 °C ± 0.8                       |
| Point T90S | Undetectable         |             |                                    |                                      |
| Point T231S | Normal                | Normal      | 74.8 °C ± 1.5                      | 73.9 °C ± 0.3                       |
| Point T515S | Normal                | Normal      | 75.6 °C ± 0.6                      | 74.6 °C ± 0.5                       |

\(^{a}\)Wild-type fluorescence and hemagglutination values are the average of nine or three determinations, respectively, using different preparations. The error given for the wild-type is the standard deviation. All other are fitting errors.

\(^{b}\)ND, not determined.
Stability and Disassembly of a Parvovirus Capsid

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