Virus Maturation Targets the Protein Capsid to Concerted Disassembly and Unfolding*

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Many animal viruses undergo post-assembly proteolytic cleavage that is required for infectivity. The role of maturation cleavage on Flock House virus was evaluated by comparing wild type (wt) and cleavage-defective mutant (D75N) Flock House virus-like particles. A concerted dissociation and unfolding of the mature wt particle was observed under treatment by urea, whereas the cleavage-defective mutant dissociated to folded subunits as determined by steady-state and dynamic fluorescence spectroscopy, circular dichroism, and nuclear magnetic resonance. The folded D75N α subunit could reassemble into capsids, whereas the yield of reassembly from unfolded cleaved wt subunits was very low. Overall, our results demonstrate that the maturation/cleavage process targets the particle for an “off pathway” disassembly, because dissociation is coupled to unfolding. The increased motions in the cleaved capsid, revealed by fluorescence and NMR, and the concerted nature of dissociation/unfolding may be crucial to make the mature particle infectious.

Viruses are macromolecular assemblies designed to exert their biological role in a single sequential cycle: 1) assembly inside the cells; 2) release to the environment; 3) attachment to new host cells; 4) disassembly and delivery of genome; and 5) replication of the genome and transcription of new viral proteins. Among these five stages, disassembly of the capsid and unpacking of the nucleic acid is the least understood (1–4). To understand the changes in stability and dynamics caused by the cleavage. With this purpose, we have utilized two baculo expressed VLPs, a wild type sequence (SFHVwt) and a mutant virus (D75N) (15). This mutant was constructed by site-directed mutagenesis of Asp75, which is at the cleavage site; replacement of the aspartic acid residue with asparagine results in the production of noninfectious particles that do not undergo cleavage (17). Therefore, the capsid of the cleavage-defective mutant is composed of 180 copies of the intact 47-kDa precursor α protein. We find that the uncleaved capsid protein can undergo dissociation not concerted with unfolding, whereas in wt VLPs, maturation cleavage favors a metastable state in which dissociation of the coat protein is coupled to unfolding. A partially folded state of the uncleaved capsid protein is characterized by fluorescence, circular dichroism, and NMR methods. In addition, cleavage may serve as part of the switch for dissociation, and the resulting “off pathway” disassembly may be important during the viral infection cycle.

**EXPERIMENTAL PROCEDURES**

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 Hepes, pH 7.0. Ultra-pure urea was obtained from Sigma.

Cells and Virus-like Particles—VLPs were expressed in Drosophila...
cells using a baculovirus expression system and purified as described previously (15). The procapsids and capsids were stored in 10 mM Ca²⁺-containing buffers.

Fluorescence Spectroscopy and High Pressure Measurements—Fluorescence spectra were recorded on an ISSK2 spectrofluorometer (ISS Inc., Champaign, IL). The high pressure cell (18) was purchased from ISS Inc. The tryptophan residues were excited at 280 nm, and emission was observed from 300 to 420 nm. Changes in fluorescence spectra at pressure $p$ were evaluated by the changes in spectral center of mass, $<v_y>$.

$$<v_y> = \sum_i F_i/\Sigma F_i$$

(Eq. 1)

where $F_i$ stands for the fluorescence emitted at wave number $v_i$. The summation is carried out over the range of appreciable values of $F_i$.

The samples were allowed to equilibrate for 15 min prior to making measurements (high pressure and urea experiments). This time was chosen because the spectroscopic changes reached a plateau within the first 10 min and did not change significantly during longer times (several hours). Unless otherwise noted, experiments were performed at 20 °C in 50 mM Hepes, pH 7.0.

Lifetime and Rotation Measurements—Lifetime and dynamic depolarization measurements were performed on a multifrequency cross-correlation phase and modulation fluorometer which uses the harmonic content of a high repetition rate, mode-locked neodymium yttrium aluminum garnet laser. This laser is used to synchronously pump a dye laser whose pulse train is frequency doubled with an angle-tuned frequency doubler (19). A detailed description of phase fluorometry lifetime and data analysis is found elsewhere (20). The quality of fits was assessed by $\chi^2$ values and by plots of weighted residuals. Excitation wavelength was 295 nm, and the emission was observed through a long wavelength pass filter (WG 335) with a cut-off at 335 nm.

Light Scattering—Light scattering was measured in an ISS 200 spectrofluorometer (21). Scattered light (320 nm) was collected at an angle of 90° to the incident light.

Far-UV Circular Dichroism—The CD spectra were obtained in 10 mM Hepes buffer, pH 7.0, using a quartz cuvette of 0.2-cm path length. Spectra were the average of two scans from 214 to 260 nm at a 100 nm/min, and the buffer and urea base lines were subtracted. Shorter wavelengths were not analyzed because of increased noise. The spectropolarimeter used was a Jasco J-715 1505 model.

Nuclear Magnetic Resonance—NMR spectra were obtained in a Bruker 600 MHz spectrometer at 25 °C. The sample was prepared in 10% D₂O using 5 mM Hepes buffer at pH 7.0. The VLP concentration was 500 µg/ml for both wt and D75N. Water suppression was achieved using the water gate sequence (22) with the composite pulse 3, 9, and 19 and a 1-ms z-pulsed field gradient at 10 G/cm. The urea peak was presaturated.

Size Exclusion Chromatography—High performance liquid chromatography was carried out in prepacked SynChromak columns (inner diameter, 250 × 4.6 mm), obtained from SynChrom Inc. (Linden, IN). GPC 500 column was used for virus elution. The system was equilibrated in 50 mM Hepes, 0.2 M sodium acetate buffer, pH 7.0, at room temperature, using a flow rate of 0.3 ml/min. Sample elution was monitored by fluorescence at 330 nm (excitation at 280 nm). The equipment used was a Shimadzu model SPD 10A.

RESULTS

Dissociation and Unfolding of FHV Capsids—The properties that stabilize a virus particle have to fulfill two apparently opposite requirements: 1) the stability must be great enough to keep the particle intact while it is outside the host cell and 2) the virus must be able to disassemble inside the host cell. The comparison between FHV virus-like particles of wild type and of the maturation-defective mutant can address the question of what is the role of maturation cleavage on particle stability. Direct reasoning suggests that maturation makes the particle more stable to the hostile environment. We measured both the dissociation of the capsids and the changes in tertiary structure of the capsid protein. Disassembly was monitored by light scattering, and denaturation was monitored by the shift in tryptophan fluorescence by changes in tryptophan lifetime and by circular dichroism. The urea-induced denaturation of wt mature capsids, as measured by fluorescence, overlapped the changes in light scattering (Fig. 2A), indicating that dissociation and denaturation are concerted events. The D75N particles disassembled at comparable urea concentrations, but the tertiary structure of the subunits required much higher urea concentrations for denaturation (Fig. 2B). These data suggest that disassembled D75N coat protein can be obtained with significant tertiary structure. In fact, at 6 M urea, there were minimal changes in solvent exposure of the D75N coat protein tryptophans (Fig. 3, A and B).

To probe the tryptophan environment in the nanosecond
show that the cleavage-defective mutant maintains a substantial mobility of the Trp residues was higher in the cleaved wt VLP obtained using a model that assigns two rotational motions for both wt and mutant VLPs, the best fit to the data was for the wt coat protein was not significantly affected by the loss of long range interactions for the wt coat protein. The fluorescence data indicate that the noncleaved mutant VLP can be readily dissociated by urea, but the coat protein retains a folded or partially folded conformation. The overall data show that the coat protein of the noncleaved D75N capsid retained substantial secondary and tertiary structure in 6 M urea. The hydrophobic core seemed to remain intact, as revealed by the fluorescence, CD, and NMR data. The fraction of dissociation in 6 M urea was the same for wt and mutant. To determine whether reassembly could occur after dissociation, gel filtration chromatography was utilized. Assembly of D75N capsid proteins (26, 27), the monomeric subunits were adsorbed to the virus, only regions with local mobility can provide sharp lines. At very low Ca\(^{2+}\) concentrations (i.e., when particles are purified in the presence of EDTA) the procapsids are readily degraded by 1% SDS or in a disassembly mixture (28), whereas 90% of the capsid (cleaved) particles survive. There is no sig-
significant difference in stability if the procapsids and capsids are exposed to 10 mM Ca\(^{2+}\) (9). In the studies reported here procapsids and capsids were stored in 10 mM Ca\(^{2+}\) containing buffers. In Fig. 2, it was shown that dissociation (measured by light scattering) was no different between cleaved and non-cleaved VLPs, whereas folding (shift in fluorescence spectra) of the wt VLP was much more susceptible to denaturation than the D75N VLP.

To examine whether the concerted dissociation and unfolding of the wt would be prevented if the final products were not free capsid proteins, high pressure experiments were performed. Hydrostatic pressure is a “clean” thermodynamic method for studying the stability of macromolecular assemblies (29–31). The main finding of the pressure studies on viruses is that the isolated capsid and the assembly intermediates assume different partially folded states in the assembly pathway. In most of the cases, pressure induces the conversion of the icosahedral particle to a ribonucleoprotein intermediate state, where the coat protein is partially unfolded but bound to RNA (26, 31–33). This ribonucleoprotein state is readily reverted to viral particles when pressure is withdrawn. Fig. 6 shows that pressure exerted reversible effects on wt and D75N VLPs. The cleavage-defective VLP was more stable against pressure than wt as measured by fluorescence (Fig. 6A) or light scattering (Fig. 6B). The reaction was almost completely reversible in both cases (Fig. 6, C and D), which agrees with previous results (27, 31) showing that pressure perturbation populates partially folded conformations of the coat protein bound to RNA that return to the native state after decompression. It is noteworthy that both wt and D75N particles eluted slightly more included after pressure treatment, probably because of reassembly into a more compact particle. The fluorescence and light scattering data overlap for wt and D75N particles. These results show that nonconcerted dissociation and denaturation of the un-

| FHV | Urea | Lifetime | Rotational correlation time (\(\theta\)) |
|-----|------|----------|--------------------------------------|
|     |      | Center Width | Average \(f_0\) \(\theta_1\) \(f_1\) \(\theta_2\) \(f_2\) |
| Wild type | 0 | 1.79 ns 1.64 | ns 9.31 11.6 0.80 0.49 0.20 |
|       | 6 | 2.70 2.94 | 4.95 9.3 0.52 0.25 0.48 |
| D75N | 0 | 1.81 1.23 | 26.9 34.9 0.77 0.19 0.23 |
|       | 6 | 1.86 1.60 | 11.3 15.8 0.71 0.37 0.29 |

The \(\chi^2\) values for the fittings of lifetime distribution and rotations were typically less than 2.0, \(f\) is the fraction of each rotational correlation time.
cleaved coat subunits only occur when they are free in solution. The greater sensitivity to pressure of cleaved wt FHV is consistent with the higher dynamics revealed by the rotation (Table I) and NMR (Fig. 4) measurements. The direct relation between protein flexibility (because of volume fluctuations) and its isothermal compressibility (34) is well established.

**DISCUSSION**

The structure of several icosahedral viruses has been determined with a remarkable level of precision (1–3). The atomic structure of viruses have revolutionized the field of virology by providing insights about the interactions of viruses with cells, drugs, and the immune system (8). However, the structures of viruses provide only frozen pictures of a final product of an assembly line. To dissect the steps of this assembly line, it is necessary to add dynamic studies to the structural information. To disassemble a virus is like dismantling the pieces of an intricate puzzle, in which the parts contain an elaborate code for self-assembly. We described here that by combining previous structural information of wild type and mutant virus-like particles of FHV with spectroscopic and hydrodynamic methods in a thermodynamic approach, it is possible to identify one of the important rules of this code. An amino acid substitution (D75N) prevents maturation cleavage of the \( \alpha \) coat protein locking the capsid as a provirion. The D75N provirion dissociates to folded coat proteins, and unfolding is not coupled to the dissociation process. On the other hand, mature wild type particles dissociate and unfold in a concerted fashion.

Nodavirus processing generates an infectious particle by cleaving the full-length \( \alpha \) subunit at residue 363 to generate \( \beta \) and \( \gamma \) subunits. The x-ray structure reveals that the first 22 residues of FHV \( \gamma \) chains are amphipathic helices that interact with RNA about the icosahedral 3-fold axes and form a helical bundle at the fivefold axes (13) (Fig. 1). Our rotation and NMR data show that it is very likely that the cleavage results in the \( \gamma \) chains gaining significant high mobility. This mobility is probably important for virus-cellular membrane interactions, perhaps in a way similar to the mechanism of fusion suggested for several enveloped viruses (35–39). Whether the \( \gamma \) chain can act as a conduit for RNA translocation is still to be proved;

**Fig. 4.** Far-UV circular dichroism and \(^1H\) NMR spectra of Flock House virus VLPs. CD spectra of wild type (A) and D75N (B) at a 100 \( \mu \)g/ml final concentration in the absence (solid line) and presence of 6 \( M \) urea (dashed line). A 0.2-cm cuvette was used. Amide region of the \(^1H\) NMR spectra of FHV wild type (C), FHV D75N mutant (D), FHV wild type in the presence of 6 \( M \) urea (E), and FHV D75N in the presence of 6 \( M \) urea (F).

**Fig. 5.** Reassembly of procapsid coat proteins into particles. FHV wild type and D75N VLPs at a final concentration of 50 \( \mu \)g/ml were incubated with 0, 6, or 8 \( M \) urea for 1 h. FHV wild type (A) and D75N (B) samples were injected in the size exclusion chromatography, and the elution profiles were monitored by the fluorescence intensity at 330 nm (excitation at 280 nm).
nated and serves as a proton donor for a hydrogen bond to the carbonyl oxygen of Asn363. Assembly locally destabilizes the protein; a, noncleaved 47-kDa coat protein; β and γ, cleaved subunits.

however, recent studies have demonstrated dramatic membrane activity of residues 1–22 of γ chain (40, 41). The mechanism for the cleavage of a subunit was proposed based on site-directed mutation studies (17). Assembly places Asp75 in a buried, hydrophobic environment resulting in an abnormally high pH of 6.8. Under physiological conditions, Asp75 is protonated and serves as a proton donor for a hydrogen bond to the carbonyl oxygen of Asn363. Assembly locally destabilizes the scissile bond between residues 363 and 364, making it susceptible to attack by a water molecule, which is probably activated by the anionic environment of the directly adjacent bulk RNA. The replacement of Asp75 with Asn prevents the peptide cleavage.

Although there was no apparent difference between wild type and cleavage-defective VLPs with regard to dissociation by urea, the concerted dissociation and unfolding of the wild type particle may render it less stable (or metastable). During infection, a trigger must exist to provoke dissociation, which would be facilitated by maturation turning the particle metastable. A coupling of unfolding to dissociation may be crucial to tunnel the activation energy and speed up the reaction.

For a given urea concentration that resulted in dissociation of wt and mutant VLPs, the yield of reassembly was much higher for the cleavage-defective particle (D75N) as measured by gel filtration chromatography. Circular dichroism confirmed the existence of a folded, dissociated D75N coat protein, whereas the CD of urea-dissociated wild type coat protein did not show any significant secondary structure. The NMR of the dissociated D75N coat protein also suggests a high molecular mass subunit, with low mobility, characteristic of a folded structure. The wt particles were less stable to pressure than the D75N mutants. The higher flexibility resulting from the cleavage of α into β + γ subunits increases the compressibility and makes the particle more sensitive to pressure. Several single-chain proteins are generally less flexible and more stable than their dimeric counterparts (42–45).

A free energy diagram (Fig. 7) sketches the dissociation of particles into folded subunits and RNA, followed by unfolding of the coat protein. For the cleaved, mature particle, the difference in chemical potential between folded and unfolded subunits (β + γ) is either nonexistent or very small. This explains the low yield of reassembly from the dissociated, cleaved proteins when urea is diluted. Overall, our results demonstrate that the maturation/cleavage process targets the particle for an off pathway disassembly, because dissociation is coupled to unfolding. Because D75N is a paradigm for a short-lived α subunit, the different properties should be crucial for the viral infection cycle. On one hand, a metastable state of the cleaved coat protein would contribute to the rapid dissociation concerted to unfolding when a specific cellular switch is turned on at the early stages of viral infection. On the other hand, after RNA replication and protein synthesis, the folded noncleaved coat protein would readily assemble into a ribonucleoprotein complex. Kinetic studies should provide additional insights about the role of metastable states in virus infectivity.

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**Fig. 6. Pressure sensitivity of wild type and cleavage-defective FHV VLPs.** The pressure stability of FHV wild type (circles) and D75N (triangles) mutant virus-like particles was analyzed by the spectral center of mass (A) and light scattering (B). To better appreciate the effect of pressure, the reaction was poised by adding 1 M urea, a subdissociating concentration. Reassembly was evaluated by size exclusion high performance liquid chromatography for wt FHV (C) and mutant after decompression (D).

**Fig. 7. Gibbs free energy diagram for the dissociation and unfolding of noncleaved (D75N mutant) and cleaved (wild type) Flock House virus.** R, RNA in the VLP; α, noncleaved 47-kDa coat protein; β and γ, cleaved subunits.
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