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Evidence of Viral Capsid Dynamics Using Limited Proteolysis and Mass Spectrometry*

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Virus particles are stable yet exhibit highly dynamic character given the events that shape their life cycle. Isolated from their hosts, the nucleoprotein particles are macromolecules that can be crystallized and studied by x-ray diffraction. During assembly, maturation and entry, however, they are highly dynamic and display remarkable plasticity. These dynamic properties can only be inferred from the x-ray structure and must be studied by methods that are sensitive to mobility. We have used matrix-assisted laser desorption/ionization mass spectrometry combined with time resolved, limited proteolysis (Cohen, S. L., Ferre-D'Amare, A. R., Burley, S. K., and Chait, B. T. (1995) *Protein Sci.* 4, 1088–1099; Kriwacki, R. W., Wu, J., Tennant, T., Wright, P. E., and Siuzdak, G. (1997) *J. Chromatogr.* 777, 23–30; Kriwacki, R. W., Wu, J., Siuzdak, G., and Wright, P. E. (1996) *J. Am. Chem. Soc.* 118, 5320–5321) to examine the viral capsid of flock house virus. Employing less than 10 μg of virus, time course digestion products were assigned to polypeptides of the subunit. Although surface regions in the three-dimensional structure were susceptible to cleavage on extended exposure to the protease, the first digestion products were invariably from parts of the subunit that are internal to the x-ray structure. Regions in the N- and C-terminal portions of the subunit, located within the shell in the x-ray structure, but implicated in RNA neutralization and RNA release and delivery, respectively, were the most susceptible to cleavage demonstrating transient exposure of these polypeptides to the viral surface.

The protein capsid of virions is a noncovalent association of protein subunits that is responsible for an array of functions, including cell attachment, cell entry, and RNA release. Mobile regions associated with these events can only be inferred from inherently static methods such as x-ray crystallography (4) and cryo-electron microscopy (5). To better understand the dynamic nature of the viral capsid it is necessary to develop methods that are sensitive to functional mobility. One recently developed method has combined limited proteolysis with mass spectrometry (1–3) to explore protein/DNA (1) and protein/protein interactions (2, 3). While previous applications of mass spectrometry to viruses have either focused on characterizing protein and DNA structure (6–11) or the intact virus (12), here we have applied the proteolysis method with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)[1] to study the surface-accessible regions of the viral particle.

MALDI-MS typically provides picomole sensitivity and accuracy on the order of 0.05% (*i.e.* ± 0.5 Da on a 1000-Da peptide) and therefore offers a useful method for identifying the proteolysis products of the virus. The limited proteolysis/MALDI-MS experiments were performed on flock house virus (FHV), a non-enveloped, icosahedral, RNA animal virus (Fig. 1) with dimensions similar to the rhino and polio viruses (~300 Å). Its protein coat or capsid is composed of 180 copies of a single gene product, protein α, which is autocatalytically cleaved to peptides, β and γ, during maturation (13). By using time-resolved proteolysis followed by MALDI-MS it was expected that the reactivities of virus particles to different proteases would be a reflection of the structure present on the surface of the viral capsid. In this study we present evidence for the transient exposure of internal viral capsid protein domains and the further use of mass spectrometry to elucidate the functional dynamics of the protein complexes (2, 3).

MATERIALS AND METHODS

MALDI-MS mass analysis was conducted using a Perceptive Biosystems Voyager Elite equipped with delayed extraction and a nitrogen laser. All analyses were conducted using 0.5 μl of 3,5-dimethoxy-4-hydroxycinnamic acid (Aldrich) in a saturated solution of acetonitrile/water (50:50) 0.25% trifluoroacetic acid. External calibration typically was accurate to 0.05% and allowed unequivocal assignment of most proteolytic fragments.

FHV was prepared in *Drosophila* cells (Schneider's line 1) suspended to 4×10^7 cells/ml in a complete growth medium containing Schneider's insect medium with 15% fetal bovine serum (CGM). FHV was added at a multiplicity of 120 plaque-forming units/cell and allowed to attach for 1 h at 26 °C. Cells were then sedimented and resuspended to 5×10^6 cells/ml in CGM. Aliquots were distributed onto 100-mm tissue culture plates and incubated at 26 °C. At 15 h postinfection, the medium was removed and monolayers were rinsed with 10 ml of ice-cold HE buffer (0.1 M HEPES (pH 7.0), 10 mM EDTA, 0.1% 2-mercaptoethanol, 0.1% bovine serum albumin). Cells were lysed in 2 ml of ice-cold HE buffer containing 1% (v/v) Nonidet p-40. Nuclei and cell debris were removed from the lysate by centrifugation for 5 min at 4 °C in a table top centrifuge. Supernatant containing the virus was pelleted through 2-ml sucrose gradients (10–30% (w/w) in HE buffer without bovine serum albumin) at 100,000 rpm for 13 min. Pellets were resuspended in 400 μl of 5 mM $CaCl_2$ 0.1% 2-mercaptoethanol buffered with HEPES (pH 7.0).

Proteolytic digests were conducted at 25 °C and 1 mg/ml virus. The endoproteases trypsin, clostripain (Arg-C), protease V8 (Glu-C), and Lys-C were purchased from Promega (Madison, WI). Endoproteinase Asp-N was obtained from Calbiochem (Table I). The activity of all the enzymes was verified with control peptides. Trypsin reactions were carried out in 25 mM Tris-HCl (pH 7.7), 1 mM EDTA. All others enzymes were in the manufacture's recommended reaction buffer. Enzyme to virus to ratio (w:w) was adjusted to 1:3000 to achieve time-resolved cleavage beginning with a sampling rate of one per minute 1 min to 24 h. Reaction volume was 10–20 μl and 0.5 μl was removed from the reaction at each time point and placed directly on the MALDI analysis

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[1] The abbreviations used are: MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; FHV, flock house virus; CPY, carboxypeptidase Y; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone.

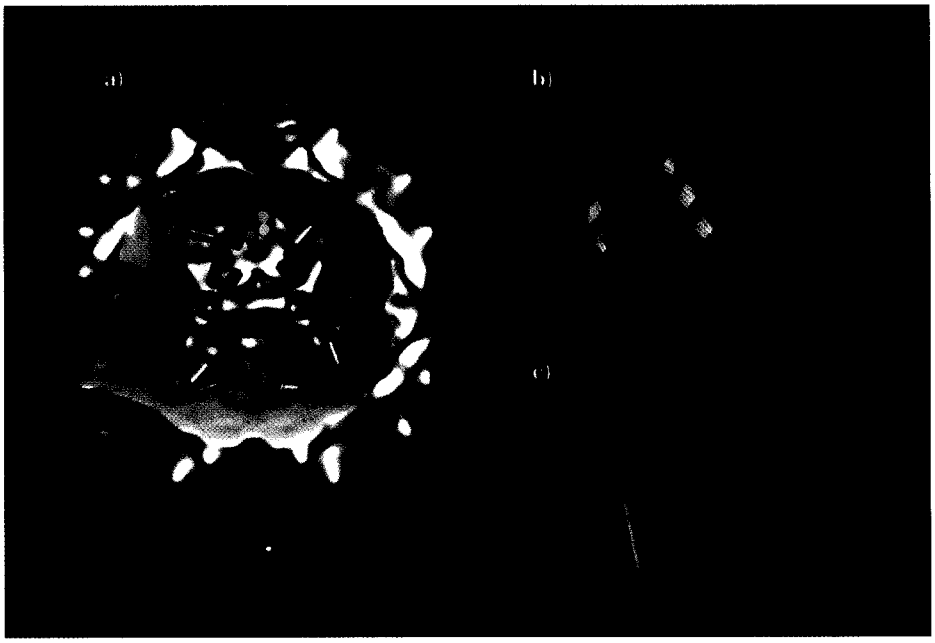

FIG. 1. **Cutaway view of the whole FHV virion.** *a*, representation of FHV based on cryo-electron microscopy and x-ray crystallography data (5). *Red, green,* and *blue* cylinders are γ-peptides. *Blue* γ-peptides are at the 5-fold axes of symmetry and form a pentameric helical bundle. *c*, close up view of the pentameric helical bundle; the 5-fold axes passes vertically through the bundle center. Two of the five β-protein monomers are shown as ribbon structures. *b*, the presence of γ-peptide on the viral surface could facilitate the release of RNA. The pentameric bundle formed by the amphipathic α-helices is of sufficient size (minimum van der Waals diameter of 20 Å) to accommodate single-stranded RNA shown in *red*.

TABLE I
Protease specificity

The limited proteolysis experiments of FHV employed five endoproteases each with different specificity's. The location of cleavage sites is denoted by a slash (/) before or after the amino acid responsible for specificity. Combinations of proteases can be used to reduce specificity and to mimic other proteases. For example Lys-C and clostripain together are specific for the same sites as trypsin.

Protease	Amino acid sequence specificity
Trypsin	*X*-K/-*X* and *X*-R/-*X*
Lys-C	*X*-K/-*X*
Clostripain	*X*-R/-*X*
Protease V8	*X*-E/-*X* and *X*-D/-*X*
Asp-N	*X*-/N-*X*

plate and allowed to dry before the addition of matrix. To confirm the identity of trypsin-released fragments the digest was then exposed to the exprotease carboxypeptidase Y or CPY (Sigma) to obtain C-terminal sequence information on each of the trypsin fragments. The trypsin/CPY sequential digests were performed by first exposing the virus to trypsin that was then inhibited with TLCK (50 μg/ml) and then allowed to dry on the MALDI sample plate. Carboxypeptidase Y (Sigma) diluted to 1 mg/ml in water was then added (1–3 μl) to the dried trypsin digest. Digestion was allowed to continue at room temperature until stopped by evaporation.

LCMS experiments were performed using an Ultrafast microprotein analyzer (Michrom BioResources, Inc. (Auburn, CA) coupled to a Perkin-Elmer Sciex API 100 electrospray mass spectrometer. Native and digested FHV were denatured prior to LCMS in a 10% trifluoroacetic acid/50% acetonitrile solution. Reverse phase LC separation used an Alpha-Chrom C18 column 2 x 150 mm (Upchurch Scientific) with a flow rate of 50 μl/min in a water/acetonitrile (0.05% trifluoroacetic acid) solvent system.

RESULTS AND DISCUSSION

Initial trypsin proteolysis experiments on FHV revealed that at suggested enzyme/protein ratios (1:10 to 1:100) numerous tryptic peptides appeared within the first minute of digestion. To better understand the kinetics and therefore accessibility of capsid domains, the reaction was slowed by lowering the enzyme concentration to an enzyme/protein ratio of 1:3000. Slowing the reaction allowed measurements to be performed at 1 min time intervals which provided greater temporal resolution. The initial trypsin cleavages were found to occur within the γ-peptide, followed by the release of peptide fragments from the N-terminal region of the β-protein and, as the digestion continued, peptides associated with B-sheets E′ and E″, which form a protruding loop on the surface of the capsid and C-terminal of the capsid protein were released (Fig. 2). As the digestion progressed, intact γ-peptide was no longer detectable. Exposure of FHV to Lys-C initially cleaved the intact γ-peptide only at one of two possible sites, and some intact γ-peptide remained present even after extended exposure (>24 h). The ability of Lys-C to only access a portion of γ-peptide is consistent with the model of FHV having T3 quasi-equivalence with two distinct "types" of the γ-peptide (4). The differential access of trypsin and Lys-C to specific cleavage sites demonstrated an unexpected specificity of the proteases to local quaternary structure. The only other observed Lys-C cleavages occurred after extended exposure at residues of the β-protein associated with loops on the viral surface and near the 5-fold symmetry axes.

Exposure of FHV to clostripain did not generate any fragments from the γ-peptide or β-protein even after prolonged exposure (>24 h). Experiments with clostripain and Lys-C, which together mimic the cleavage specificity of trypsin, resulted in fewer cleavages on the FHV viral capsid than those with trypsin. Because clostripain is unable to access putative cleavage sites before and after treatment with Lys-C, these results further suggest that the initial cleavages must not cause large-scale unfolding of the capsid proteins. These results are consistent with previous experiments where access of proteases (14) and nucleases to the interior of viruses is known to be limited by the failure of nucleases to cleave viral nucleic acids. Exposure of FHV to the endoprotease Glu-C resulted in initial cleavage of the γ-peptide. Likewise, Glu-C cleaved the β-protein at sites consistent with amino acids located on the surface and edges of the capsid monomer after prolonged exposure. Exposure of FHV to Asp-N generated no fragments, even though putative cleavage sites are present on the β-protein.

Interestingly, electron micrographs of the viral particles revealed that they maintained their spherical shape even after exhaustive enzyme digestion, yet when analyzed under the denaturing conditions of SDS-polyacrylamide gel electrophoresis no intact β-protein was detected. This result indicated that even though quantitative cleavage had occurred the viral particle maintained its structural integrity. To further confirm that the results obtained reflected a population-wide digestion of the γ-peptide, high performance liquid chromatography-

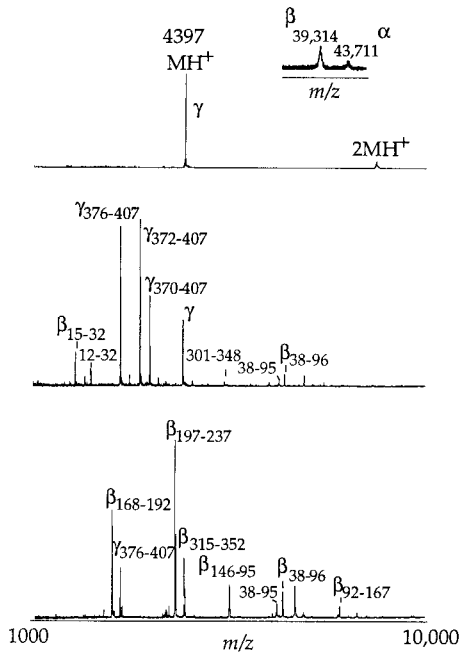

FIG. 2. **MALDI-MS data generated from the trypsin digest of FHV.** *Top*, native FHV. The capsid protein undergoes an autocatalyzed cleavage event in most of its subunits during maturation. The precursor α-protein and products, β-protein and γ-peptide, were detected. *Middle*, 15 min after the addition of trypsin the γ-peptide and three fragments were observed along with proteolytic fragments from β-protein. *Bottom*, after 24 h of exposure the γ-peptide exists only as the uncleavable fragment 376–407. The ion of highest intensity contains the loop region of the β-protein present on the viral capsid surface. All digests were performed on FHV at 1.0 mg/ml and at 25 °C.

TABLE II

Sequential digestion of FHV with trypsin and CPY

Tryptic digestion was stopped with the inhibitor TLCK, and 0.5 μl was transferred to the MALDI-MS analysis plate. CPY was added directly to the dried sample well and digestion proceeded during evaporative drying, generating a ladder sequence that allowed the unequivocal identification of proteolytic fragments.

Trypsin digestion	CPY sequential digestion
γ-Peptide (364–407)	γ-Peptide (364–406)
γ-Peptide (370–407)	γ-Peptide (370–406)
γ-Peptide (372–407)	γ-Peptide (372–406)
	γ-Peptide (372–405)
	γ-Peptide (372–404)
γ-Peptide (376–407)	γ-Peptide (376–406)
	γ-Peptide (376–405)
	γ-Peptide (376–404)
β-Peptide (12–32)	β-Peptide (12–31)
β-Peptide (15–38)	β-Peptide (15–37)
	β-Peptide (15–36)
	β-Peptide (15–35)

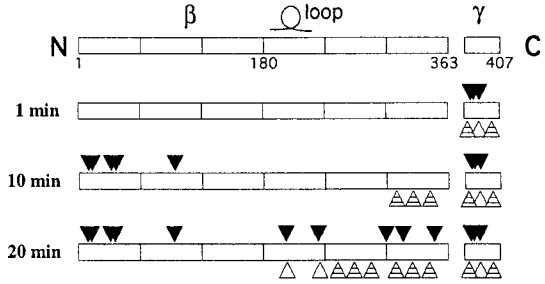

FIG. 3. **Proteolytic cleavage sites mapped to the FHV capsid protein.** The kinetics of the proteolysis reaction are demonstrated in this time course experiment. The γ-peptide and the N and C terminus of β-protein are domains that are localized internally. Cleavages localized to the capsid surface (loop domain) are not initially present. The cleavage sites of trypsin, Lys-C, and Glu-C, are represented by *black*, *white*, and *striped arrows*, respectively.

mass spectrometry analysis was performed before and after digestion. Here UV and mass spectral data for the γ-peptide was obtained before digestion and was absent after trypsin treatment. Quantitative MALDI-MS experiments were also employed using a synthetic γ-peptide fragment as an internal standard; the results of these experiments produced a linear correlation between the internal standard and γ-peptide. The calibration of the γ-peptide against the γ-peptide fragment was then used to monitor γ-peptide content as a function of exposure to digestion. These experiments also confirmed the population-wide digestion of the γ-peptide. To assure that correct assignments of proteolytic fragments were made, a "sequential digestion approach (endo- followed by exoproteolysis) followed by MALDI-MS analysis (15) was used to further characterize the proteolytic fragments (Table II). In cases where complicated mixtures precluded the use of sequential digestion, HPLC purification and Edman sequencing were employed.

As a group the time course experiments clearly demonstrated that the kinetics of proteolysis are domain-specific (Fig. 3). Many of the cleavage sites were consistent with the expected surface structure of the virus such as the surface loop digestion, yet the kinetics of cleavage of the γ-peptide and the N-terminal of the β-protein revealed that sites internal in the x-ray structure were the most susceptible. These experiments indicate that the γ-peptide and the N-terminal region of the β-protein, which are crystallographically defined as being internal to FHV, are part of the solvent-accessible domain. When combined with the crystal structure data, the proteolysis results strongly suggest that these domains are transiently present on the viral surface.

Previous experiments with poliovirus offer precedent for these observations. Binding experiments with poliovirus demonstrated that monoclonal antibodies specific to internal domains of capsid proteins VP1 and VP4 could reversibly neutralize the virus (16). Additional experiments (17) on poliovirus with proteases, antiviral monoclonal antibodies, and antisera against synthetic peptides also suggest that externalization may occur in solution. The exposed amino terminus of VP1 was also shown to be responsible for liposome attachment. These previous studies, and our results from FHV, are consistent with a dynamic capsid in which specific protein regions can translocate to the capsid surface.

In conclusion, previous studies on FHV (5, 18) suggested that the γ-peptides are involved in the release of RNA via a dynamic mechanism where γ-peptides, in an amphipathic α-helical configuration, form a pentameric helical bundle at the 5-fold symmetry axes creating a conduit for the release of RNA. Our proteolysis experiments now confirm that γ-peptides are in equilibrium with the capsid surface. In addition, the mass spectrometry-based method for structural analysis offers information that is complementary to crystallographic and electron microscopy data. Since the MALDI/proteolysis approach does not require the formation of crystals, consumes only microgram quantities of material, and can unequivocally identify proteolytic cleavage sites, it offers a unique and efficient approach to viral structural analysis. Most importantly, since the proteolysis is performed in solution, this method can significantly contribute to an understanding of the dynamic domains that initiate RNA release and translocation, the final phase of the virus life cycle.

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REFERENCES

1. Cohen, S. L., Ferre-D'Amare, A. R., Burley, S. K., and Chait, B. T. (1995) *Protein Sci.* **4,** 1088–1099
2. Kriwacki, R. W., Wu, J., Tennant, T., Wright, P. E., and Siuzdak, G. (1997) *J. Chromatogr.* **777,** 23–30
3. Kriwacki, R. W., Wu, J., Siuzdak, G., and Wright, P. E. (1996) *J. Am. Chem. Soc.* **118,** 5320–5321
4. Fisher, A. J., and Johnson, J. E. (1993) *Nature* **361,** 176–179
5. Cheng, R. H., *et al.* (1994) *Structure* **2,** 271–282
6. Gorman, J. J., Corino, G. L., and Shiell, B. J. (1990) *Biomed. Environ. Mass Spectrom.* **19,** 646–654
7. Gorman, J. J. (1992) *TRAC Trends Anal. Chem.* **11,** 96–105
8. Winkler, M. A., Kundu, S., Robey, T. E., and Robey, W. G. (1996) *J. Chromatogr. A* **744,** 177–185
9. Jonscher, K. R., and Yates, J. R. (1997) *J. Biol. Chem.* **272,** 1735–1741
10. Jurinke, C., *et al.* (1996) *Genet. Anal.* **13,** 67–71
11. Despeyroux, D., Phillpotts, R., and Watts, P. (1996) *Rapid Commun. Mass Spectrom.* **10,** 937–941
12. Siuzdak, G., *et al.* (1996) *Chem. Biol.* **3,** 45–48
13. Zlotnick, A., Reddy, V. S., Dasgupta, R., Schneemann, A., Ray, W. J., Rueckert, R. R., and Johnson, J. E. (1994) *J. Biol. Chem.* **269,** 13680–13684
14. Gallagher, T. M., and Rueckert, R. R. (1988) *J. Virol.* **62,** 3399–3406
15. Nelson, R. W., Dogruel, D., Krone, J. R., and Williams, P. (1995) *Rapid Commun. Mass Spectrom.* **9,** 1380–1385
16. Li, Q., Yafal, A. G., Lee, Y., Hogle, J., and Chow, M. (1994) *J. Virol.* **68,** 3965–3970
17. Fricks, C. E., and Hogle, J. M. (1990) *J. Virol.* **64,** 1934–1945
18. Munshi, S., *et al.* (1996) *J. Mol. Biol.* **261,** 1–10