Capsid Assembly in a Family of Animal Viruses Primes an Autoproteolytic Maturation That Depends on a Single Aspartic Acid Residue*

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Maturation of noninfectious nodavirus provirions occurs by autoproteolytic cleavage of most of the 180 copies of the α-protein that make up the icosahedral capsid. This maturation, which is much slower than viral assembly, produces an infectious particle that is more stable than the provirion and makes viral uncoating thermodynamically distinct from assembly, allowing assembly and (a time-delayed) uncoating to occur under similar conditions. The results of structural, computational, and molecular genetic studies suggest that maturation depends on intrasubunit strain, produced during assembly, and on a single aspartic acid residue. This residue lies in a hydrophobic pocket that is stabilized by intersubunit contacts. It is close to the scissile bond and exhibits an environmentally elevated pKₐ. The apparent involvement of a single acidic residue in the hydrolytic cleavage of a peptide bond contrasts with the involvement of 2 such residues in acid proteases.

The nodaviruses are a family of small icosahedral viruses infecting insects, mammals, and fish (1–3). They are among the simplest of all animal viruses. The virus particle consists of 180 copies of the coat protein, which encapsidates the bipartite RNA genome. Only three proteins are encoded in the viral genome. The α-protein (the coat protein precursor) is encoded on RNA2; RNA1 encodes a replicase and a small protein of unknown function (1, 2). The simple particle and genome have made this system an attractive subject for the study of viral capsid structure and assembly as well as viral gene expression. Like the more common and complex picornaviruses (which include important pathogens such as poliovirus, rhinovirus, and foot-and-mouth disease virus) (4), nodaviruses are initially constructed as provirions, which mature to an infectious virion by post-assembly cleavage (5). The simple particle and genome have made this system an attractive subject for the study of viral capsid structure and assembly as well as viral gene expression.

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Nodavirus capsids display T = 3 icosahedral symmetry; the 60 icosahedral asymmetric units contain three copies of the α-protein, each in a slightly different, quasi-equivalent environment (see Fig. 1A) (6). The full-length coat protein of Flock House virus (407 amino acids) assembles rapidly in vivo (within 5 min of synthesis), followed by a slow autoproteolytic cleavage of most subunits (5). The 363-residue NH₂-terminal fragment (β-protein) forms the virus capsid; the 44-residue COOH-terminal fragment (γ-peptide) remains associated with the interior of the capsid. Maturation proteolysis is required for infectivity (7) and results in a marked increase in virion stability (5). At present, structural information is available only for nodaviruses in the cleaved mature state (8–10). The recently refined 2.8-Å structure of black beetle virus (10) has provided us the opportunity to examine the cleavage site in greater detail.

MATERIALS AND METHODS

Structural Observations—Black beetle virus was crystallized in 50 mM sodium phosphate, 0.55 M ammonium sulfate, 1% polyethylene glycol 8000 (Sigma) at pH 7 (11). The structure was solved by multiple isomorphous replacement and molecular averaging (8). The 2.8-Å structure of the virus, including 10 ribonucleotides of the RNA/icosahedral asymmetric unit, was refined to a final R-factor of 22.1% as described by Wery et al. (10).

Development of Model α-Protein and Electrostatic Calculations—the procedure of Yang et al. (12) was followed to calculate the pKₐ of Asp-75 using the atomic coordinates from the refined structure of black beetle virus (10) with hydrogen atoms generated by CHARMM (13). To model the uncleaved α-protein, the COOH terminus of the β-protein and the NH₂ terminus of the γ-peptide were explicitly connected and then subjected to energy minimization by CHARMM (13). Amino acids within a 20-Å radius of the cleavage site, including all hydrogens, were used in this calculation (100 steps of Powell minimization; harmonic constraints applied to the positions of non-hydrogen atoms). Final coordinates of the non-hydrogen atoms, including the NH₂ terminus of the γ-peptide, were shifted by 0.3 Å r.m.s. from the refined crystallographic coordinates (10).

The resultant structure, with CHARMM partial charges (all atoms included) on the respective atoms, was used to calculate the pKₐ shifts by numerically solving a linearized Poisson-Boltzmann equation by the finite difference method with the program DELPHI (DELPHI Version 3.0, courtesy of Barry Honig, Columbia University) (14). Calculations were done with a grid spacing of ~0.4 Å. All bound waters were considered explicitly as part of the protein. The dielectric constants of the solvent and protein were set to 78.5 and 4.0, respectively, and the ionic strength was 0.15 M.

pH Profile of Autoproteolysis—Drosophila cells (Schneider's line 1) were suspended to 4 × 10⁴ cells/ml in a complete growth medium containing Schneider's insect medium with 15% fetal bovine serum (CMG). Flock House virus was added at a multiplicity of 120 plaque-forming unit/cell and was allowed to attach for 1 h at 26 °C. Cells were then sedimented and resuspended to 5 × 10⁶ cells/ml in CMG. 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 25 mM PIPES (pH 6.8), 100 mM NaCl, 0.1% BSA.

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1 The abbreviations used are: r.m.s., root mean square; PIPES, 1,4-piperazinediethanesulfonic acid; BSA, bovine serum albumin.
Cells were then covered with 5 ml of methionine-deficient Grace's insect medium (Life Technologies, Inc.) with 59 μCi of [35S]methionine (Amersham Corp.).

After 1 h of incubation at 26 °C, monolayers were rinsed with 10 ml of ice-cold HE buffer (0.1 M HEPES [pH 7), 10 mM EDTA, 0.1% 2-mercaptoethanol, 50% BSA). Cells were lysed in 1 ml of ice-cold lysis buffer containing 1% (v/v) Nonidet P-40. Nuclei and cell debris were removed by centrifugation for 5 min at 4 °C in a tabletop centrifuge (Sorvall Microspin 24S). Virus (in 1 ml of supernatant) was pelleted through 2-ml sucrose gradients (10-30% (w/w) in HE buffer without BSA) at 100,000 r.p.m. for 13 min at 4 °C in a TLA-100.3 fixed angle rotor (Beckman Instruments). Pellets were resuspended in 400 μl of a solution of 5 mM MgCl₂, 5 mM CaCl₂, 0.1% 2-mercaptoethanol buffered with a 0.1 M concentration of one of the following: sodium citrate (pH 3 or 4), sodium acetate (pH 5), HEPES (pH 7), or Tris-HCl (pH 8 or 9).

Freshly isolated [35S]methionine-labeled provirions, resuspended in buffers at different pH values, were incubated at room temperature. Samples (30 μl) were withdrawn immediately after resuspension and 3, 6, 5, and 24 h later. The samples were mixed with an equal volume of 2 x electrophoresis buffer and stored frozen until analysis on 12% SDS-polyacrylamide gel (15). Gels were fixed and exposed to Kodak X-AR5 film. The extent of α-protein cleavage was quantified by densitometry using a Zenith densitometer.

Mutagenesis and Analysis of Mutants and pH Profile of Cleavage—Conditions for site-directed mutagenesis, transfection of Drosophila cells, [35S]methionine labeling, purification of virus particles, and gel electrophoresis were as described (17). Briefly, 200 μg of purified virion RNA1 plus 200 μg of RNA2 transcripts were used to transfect 10⁷ Drosophila cells. After 15 h, 50 pCi of [35S]methionine was added to the medium, and incubation was continued for another hour at 26 °C. Virus particles were purified by pelleting the cell lysate through a 30% (w/w) sucrose cushion, followed by a 5-20% (w/w) sucrose gradient. Peak fractions were pooled and incubated for 24 h at room temperature to allow cleavage (5). Viral proteins were separated on 12% SDS-polyacrylamide gel (15). The gel was fluorographed after treatment with Amplify (Amersham Corp.).

RESULTS AND DISCUSSION

X-ray Structure of Mature Particle (β + γ)—The sites of maturation cleavage in the three identical gene products that form theicosahedral asymmetric unit of black beetle virus lie close to subunit interfaces and are near the internal surface of the shell (Fig. 1, A and B). The environments of each of the three unique cleavage positions are very similar, with a r.m.s. deviation for coordinates of residues shown in Fig. 2 (A and B) and for α-carbons within 15 Å of the cleavage site. The COOH terminus of the β-protein (Asn-363) lies within a hydrophobic pocket, and the NH₂-terminal amine of the γ-peptide (Ala-364) is exposed to the aqueous environment in the interior of the virus, 8.9 Å from the COOH-terminal carbon of the β-protein for the C subunit (Fig. 2, A and B). Neither residue is accessible to exogenous proteases. In the mature virion, the carboxyl group of the cleavage-produced COOH-terminal Asn-363 and the side chain of Asp-75 share a proton (O-H-O; 2.5 Å).

Model of α-Protein—The mechanism for the cleavage-induced maturation of nodaviruses proposed here is based on observations of the mature subunit and a model of the intact α-protein in the procapsid. The latter was reconstructed from the refined x-ray model of the cleaved β- and γ-proteins in the mature crystalline virus. In the reconstructed α-protein, the portion corresponding to β is essentially unchanged from its position in the mature protein (overlapping r.m.s. movement of 0.4 Å for all residues within 15 Å of the cleavage sites in the three subunits). By contrast, the r.m.s. shifts in the positions of the amino-terminal residues of the γ-protein are 2.7, 0.9, and 0.3 Å for residues 364-366, respectively, averaged over the three subunits. This model of α-protein suggests that the separation between Asn-363 and Ala-364 caused by the autoproteolytic maturation can largely be accounted for by local movement of the amino terminus of the γ-protein and a minimal change in Asn-363. This conclusion seems reasonable, partly because the observed interactions of the carboxyl-terminal residue of the β-protein (Asn-363) with Asp-75, Tyr-176, and Ala-360 (Fig. 2B), each of which is conserved in the four characterized nodaviruses (16), can be maintained in the proposed model of the α-protein. The amino-terminal residue of the γ-peptide in the mature virus interacts less extensively with adjacent residues. In addition, major structural changes would be required to form the α-protein by maintaining the NH₂-terminus of the γ-peptide and moving the COOH-terminal segment of the β-protein into position where a peptide bond could be formed between Asn-363 and Ala-364. Hence, there is good reason to believe that the conformation of the COOH-terminal portion of the cleavage site in the mature β-protein is very similar to that in the α-protein of the procapsid.

Cleavage Mechanism—The mechanism that we have proposed for the cleavage reaction depends on four features observed in the x-ray structure and/or in the model of the α-protein. (a) Hydrogen bonds formed by the amide side chain of Asn-363 direct the carbonyl oxygen toward Asp-75 (Fig. 2B). The functional groups that interact with Asn-363 and Asp-75 (Fig. 2B) are conserved within the nodaviruses family (16). (b) Asp-75 is at least partially protonated in the α-protein. The environmental effects of the hydro-
**Assembly-dependent Autoproteolytic Virus Maturation**

Fig. 2. Structure of solvent-ocluded cleavage site. A, the electron density map and the refined molecular model of the cleavage site of the C subunit of black beetle virus crystallized at pH 7.0 (11). The relationship between the COOH-terminal carboxylic acid of the β-protein and the side chain of Asp-75 (red) is accentuated by the close proximity of the corresponding electron density. The cleavage products, the COOH-terminal carboxylic acid of the γ-protein, and the NH-terminal amine of the γ-peptide are in green. Although density from the C subunit is shown, the structures of the cleavage sites of all three subunits of black beetle virus are very similar (10). B, the molecular model highlighting the network of hydrogen bonds that direct interactions between Asn-363 (the COOH terminus of the β-protein) and Asp-75. The hydrophobic amino acids around the cleavage site are shown are blue. Functional groups that participate in hydrogen bonding interactions are conserved within the nodavirus family (12). Asn-363 is the last residue in a type 1 β-turn, where both the side chain and peptide amides donate hydrogens to the carbonyl of Ala-360. The side chain carbonyl of Asn-363 accepts a hydrogen bond from the phenolic hydroxyl of Tyr-176. Tyr-176 is on the D strand of the coat protein subunit's β-barrel. These interactions direct the COOH terminus of Asn-363, created by proteolysis, toward the side chain carboxylic acid of Asp-75, forcing protonation of one or both of the two acids at physiological pH and formation of a hydrogen bond. The COOH-terminal carboxyl also accepts a hydrogen bond from the peptide amide of Ser-365 of the γ-peptide. Asp-75 accepts a hydrogen bond from the peptide amide of Met-366. The black beetle virus structure has been refined to an R-factor of 21%; the extraordinary similarity between quasi-symmetrically related subunits is investigated in greater detail elsewhere (10). This figure was generated using MACINPLOT (21) and FRODO (22).

Phosphorylated cleavage site, including the lack of an identifiable counterion and the peptide carbonyl acting as a hydrogen bond acceptor, are expected to raise the free energy of ionization (and the pK) of Asp-75 (17, 18). (c) Protonated Asp-75 acts as a general acid, polarizing the main chain carbonyl of Asn-363 and making it susceptible to nucleophilic attack by a water molecule (Fig. 3A). The active water molecule is incorporated into the COOH-terminal carboxylic acid of the β-protein and cannot be identified in the structure of the mature virus. In a hydrophobic environment, like the cleavage site, where a proton cannot be readily transferred, general acid catalysis may be an important hydrolytic mechanism. It is also possible that interaction of Asp-75 with the Asn-363 peptide carbonyl may activate proteolysis by destabilizing the geometry of the scissile peptide bond. (d) The increased stability of the mature capsid is thermodynamically linked to cleavage and helps drive proteolysis. The absence of cleavage prior to assembly suggests that quaternary interactions initiate autoproteolytic maturation. Provirion assembly probably has a role in stabilizing the hydrophobic cleavage site (altering the pK of Asp-75) and adding strain to the scissile bond of Asn-363–Ala-364.

To test the above hypothesis, the protonation state of Asp-75 was analyzed using a linearized Poisson-Boltzmann equation and estimated solvation energy as implemented in the program DELPHI (14). The calculations were based either on the model of the uncleaved α-protein or on the 2.8-Å refined coordinates of
black beetle virus modified to eliminate the charge on the COOH terminus of the β-protein. In both model systems, the presence of Asp-75 is elevated to pH -6.0 in all three quasi-equivalent sites. Thus, a significant fraction of Asp-75 is protonated near pH 6, the optimal pH of the maturation cleavage reaction in Flock House virus (data not shown).

The plausibility of this mechanism was tested by site-directed mutagenesis of Asp-75 in Flock House virus. The coat proteins of black beetle virus and Flock House virus share 87% identity. Residues near the cleavage site are more strictly conserved (16). Replacement of Asp-75 with glutamic acid (D75E), asparagine (D75N), threonine (D75T), or valine (D75V) resulted in the production of noninfectious particles that did not undergo maturation cleavage (Fig. 4). The D75N mutant is an asparagine residue, while the D75E mutant retains the carboxylic acid of the wild-type protein, but alters the relative position of the catalytic group. Drosophila cells were transfected with purified Flock House virus RNA1 plus mutant transcript RNA2 to initiate one round of infection. Plaque assays showed that progeny were not infectious within the detectable limit (<0.1% of the wild-type transfection). The loss of infectivity correlated with the absence of maturation cleavage. After 24 h at room temperature, which is sufficient for 90% cleavage of the wild-type α-protein, only uncleaved protein was observed in purified mutant capsids (Fig. 4).

When Asn-363, which positions the scissile bond with respect to Asp-75 (Fig. 2B), was replaced by alanine or aspartic acid, the synthesis of noninfectious, cleavage-defective particles (7) was again observed. The mutant N363T does undergo cleavage under some conditions, but the cleavage is drastically slowed down under all conditions.

**Cleavage Kinetics: Subunit Communication or Quasi-equivalent Environments?**—Maturation kinetics of Flock House virus do not follow a simple first-order rate law (5), as expected for an intramolecular reaction, since the cleavage rate slows more rapidly than would be expected for such a reaction. If the cleavage reaction at a given site depends on a local strain, the cleavage rate slows more rapidly than would be expected for such a reaction. If the cleavage reaction at a given site depends on a local strain, the cleavage rate slows more rapidly than would be expected for such a reaction. If the cleavage reaction at a given site depends on a local strain, the cleavage rate slows more rapidly than would be expected for such a reaction. If the cleavage reaction at a given site depends on a local strain, the cleavage rate slows more rapidly than would be expected for such a reaction. If the cleavage reaction at a given site depends on a local strain, the cleavage rate slows more rapidly than would be expected for such a reaction.

![Diagram](https://via.placeholder.com/150)

**FIG. 3.** Proposed mechanism for autoproteolytic maturation of nodavirus capsid protein results from chemical (A) and thermodynamic (B) factors affected by assembly and maturation. A. Asp-75 is highly protonated even at neutral pH by virtue of its burial in a hydrophobic environment stabilized by the association of subunits during assembly (1). It forms a hydrogen bond with the carbonyl of the Asn-363–Ala-364 peptide bond, and this is sufficient to make it susceptible to nucleophilic attack by water (2) to form a tetrahedral intermediate (3). The water molecule is not trapped within the cleavage site, but probably originates from within the hydrophilic interior of the virus capsid. The intermediate can relax by loss of the amine from the nascent peptide (4, 5), yielding the hydrolyzed peptide bond at the cost of one water molecule (6). The hydrophobic protonation/activation of a catalytic acid residue resembles the activation of the catalytic Asp in lysozyme (23). Conversely, in the typical acid protease, it is the close interaction between 2 aspartates that is responsible for the elevated pK of one of the catalytic acids. B, shown is a scheme describing the thermodynamics of virus assembly and maturation. The cartoon relates the activation and relaxation of the coat protein subunit in the course of capsid assembly and maturation. The free energy of activated α (α-protein in the provirus, but not including the stabilizing free energy from quaternary interactions) is greater than that of the free α-protein by ∆G1, and this energy may stabilize an intermediate in the cleavage. Quaternary interactions stabilize formation of the relatively unstable provirus favoring spontaneous assembly. Autoproteolytic maturation is energetically driven by ∆G2; the combination of energy gained by relaxation of the strained provirus conformation of the coat protein and the change in the free energy of intersubunit interactions in the mature capsid. Although the provirus and mature particle are drawn as substantially different structures, the two particles are nearly identical in their physical parameters (sedimentation coefficient and diameter). The gain in particle stability is accompanied by a very subtle change in quaternary structure.

![Electropherogram](https://via.placeholder.com/150)

**FIG. 4.** Electropherogram showing cleavage phenotype of intact virus particles purified from Drosophila cells transfected with viral RNA. Maturation cleavage of the α-protein to the β-protein is seen in the wild-type virus (D75L/FHV). Only the α-protein is observed when Flock House virus Asp-75 is mutated to valine (D75V), glutamate (D75E), asparagine (D75N), or threonine (D75T). The γ-peptide (5 kDa) is not visible on this gel. The identity of bands migrating near the 14-kDa marker is not known.
rate of cleavage will decrease more rapidly than for a first-order process.

\[\frac{d[a]}{dt} = -k(a)/[a^*][a]\]  

(Eq. 1)

In a reaction whose progress is described by this rate law, the effective rate constant for the cleavage of the α-protein \((k(a)/[a^*])\) will decrease from an initial value of \(k\) at \([a^*][a]=1\) to essentially zero when the fraction of uncleaved α-protein approaches zero. The cleavage kinetics observed by Gallagher and Rueckert (5) can be described by Equation 1, where \(k = 0.315\) h\(^{-1}\), which would correspond to a half-time of 2.2 h if the effective rate constant for the reaction remained unchanged throughout. In the simplest case, shown here, the same rate constant \((k)\) would apply to all three classes of subunits in the provirus. In addition, the progressive gain in stability would have to be distributed uniformly over the entire capsid so that, in effect, the cleavage rate on one side of the virus would be affected by the cleavage of a subunit on the other side.

Alternatively, the multiphasic first-order kinetics can be interpreted as an effect of the \(T = 3\) geometry (Fig. 1A), where 120 subunits cleave with a fast rate \((t_{1/2} = 2.2\) h\) and 60 subunits cleave with a slow rate \((t_{1/2} = 13.1\) h\) (5). This interpretation suggests that the scissile Asn-Ala bond in two classes of subunit is cleaved at nearly the same rate and that one of the three classes (A, B, or C) cleaves more slowly. In fact, the A and C subunits do possess features that distinguish them from the B subunit. The C subunit is located near the vertex of an icosahedral 2-fold axis (Fig. 1B); the protein loop that includes Asp-75 interacts with the peptide in the groove coming from the same C subunit. On the other hand, the γ-peptide of the A subunit is in a distinctly different environment than those of the B and C subunits. The five A subunit γ-peptides around each icosahedral 5-fold axis form a five-helix bundle (10).

Unfortunately, these two extreme cleavage models cannot be distinguished easily by kinetics since both fit the data well at \(t < 24\) h. Although these models predict different behavior at much longer time intervals, an accurate assessment of reaction kinetics after several half-times is very difficult due to contributions that could arise from an even slight heterogeneity of the sample. And it goes without saying that more sophisticated models (involving a more localized intersubunit communication or a combination of intersubunit communication and different initial rates for quasi-equivalent subunits) require too many variables to be tested critically. In spite of this ambiguity, we strongly favor the model based on intersubunit communications since at very long time intervals, i.e. weeks to months, a few percent of α-chains remain. Although one could argue that the uncleaved α-chains arise from a minor noninfectious population of defective provirions that do not undergo cleavage or from a population of defective provirions that undergo only a limited cleavage, we discount this possibility on the basis that intersubunit communication has also been implicated in the autoproteolytic maturation of hepatitis A (19), a picornavirus. Bishop and Anderson (19) point out that cleavage of the 60 copies of the precursor protein VP0 to the mature proteins VP4 and VP2 is linear with time, i.e. it does not follow simple first-order kinetics. In fact, the observed kinetics suggest that early cleavage events accelerate subsequent cleavage. This rate-enhancing effect contrasts with the rate-attenuating effect proposed for the nodaviruses. It is likely that the chemical details of picornavirus cleavage differ from those of nodavirus cleavage. It is important that, in both virus families, there appears to be a cooperative effect influencing cleavage as reflected by the failure to follow first-order kinetics for what should be first-order reactions.

In summary, we believe that assembly-based instability of the viral capsid and the alteration of the pK\(_a\) of Asp-75 provide an attractive thermodynamic and chemical basis for the observed cleavage of a normally inert peptide during viral maturation. We hypothesize that maturation-induced cleavage will occur efficiently only within a provirus complex in which there are localized "high energy" regions and the protonated aspartic acid. If such regions do exist and if they contribute to post-assembly modification of the coat protein, viral uncoating would become thermodynamically distinct from viral coating, rather than simply the reverse of the coating process. Thus, in the life cycle of the virus, it seems reasonable that both assembly and disassembly should be thermodynamically favorable under the conditions where each occurs and that both may occur under similar conditions. In addition, it may be advantageous for the disassembly process to involve a pair of β- and γ-proteins, where γ may be able to dissociate from the particle during the uncoating process (24).

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