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Proteolytic Dissection of Sindbis Virus Core Protein

ROLAND K. STRONG† AND STEPHEN C. HARRISON*

Harvard University and Howard Hughes Medical Institute, Fairchild Biochemistry Building, 7 Divinity Avenue, Cambridge, Massachusetts 02138

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Mild trypsin treatment of the Sindbis virus nucleocapsid protein yields a fragment with a molecular mass of ~18.5 kilodaltons with its N terminus at residue 105. The fragment, which is stable to further digestion, appears by gel exclusion chromatography to be monomeric. These data are consistent with a model for the alphavirus core proteins, consisting of an extended and flexible N-terminal arm (residues 1 to 103) and a compactly folded C-terminal domain (residues 104 to 274), as previously suggested on the basis of sequence characteristics.

Sindbis virus is a simple, enveloped spherical virus ~70 nm in diameter (7, 13). The outer surface is composed of glycoprotein heterodimers, which span the membrane bilayer, arranged with icosahedral symmetry on a T=4 lattice as shown by negative stain and rotary-shadowed electron microscopy and more recently by image reconstruction from cryoelectron micrographs of virions in vitreous ice (4, 7, 14). The fenestrated nucleocapsid core particle (~40 nm in diameter) displays T=3 icosahedral symmetry (4, 5). The sequence of the nucleocapsid protein (termed C; 264 residues, 30 kilodaltons [kDa]) suggests a molecular organization similar to that of the coat proteins of the T=3 plant viruses (12). The N-terminal 103 residues are unusually rich in arginine, lysine, and proline; the remainder of the molecule has a conventional amino acid composition (12). On the basis of sequence alignments with picornavirus VP3 capsid proteins, Fuller and Argos have proposed that this C-terminal portion folds into the β-barrel characteristic of the structural proteins of other positive-stranded RNA viruses (5). We report here the results of experiments with proteolysis of intact and dissociated Sindbis virus cores consistent with this view of the modular organization of the core protein.

Preparation of Sindbis virus. Sindbis virus, either a heat-resistant or a wild-type isolate, was prepared by a variation of the methods of Bell et al. and Burge and Pfefferkorn (1, 2). The buffer used throughout was 25 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)]–150 mM NaCl–0.1 mM EDTA (PNE; pH 7.4).

Preparation of nucleocapsid cores. Triton X-100 (Surface-Amps grade; Pierce Chemical Co.) or *n*-octyl-β-D-glucopyranoside (Calbiochem-Behring) was added to a Sindbis virus solution (~6 mg/ml in 25 mM Tris–150 mM NaCl–0.1 mM EDTA [TNE; pH 7.4]) to a final detergent concentration of 1% (wt/wt) and incubated at 4°C for 15 min. Cores were pelleted by layering 500 μl of this solution onto a two-layer step gradient consisting of 1.3 ml of 5% (wt/wt) sucrose and 0.5% (wt/wt) detergent-TNE on top of 3.5 ml of 10% (wt/wt) sucrose-TNE which was subsequently spun at 45,000 rpm for 90 min in an SW 50.1 rotor (Beckman Instruments, Inc.). The cores were rinsed free of sucrose by suspending the pellet in TNE, repelleting it in a Beckman Airfuge, and resuspending it to ~15 mg/ml with TNE. The solutions used during the preparation of cores and all glassware were treated with diethyl pyrocarbonate (Sigma Chemical Co.) and autoclaved to inactivate RNases. Cores prepared by this method in Triton X-100 were ≥95% pure and very uniform, as judged by electron microscopy; the yield was ~80%. However, cores prepared with *n*-octyl-β-D-glucopyranoside were of lower purity, homogeneity, and yield.

Proteolysis of cores by an endogenous protease activity. Cores prepared from virus treated with Triton X-100 were stable at 4°C for several weeks; those produced with *n*-octyl-β-D-glucopyranoside were observed to undergo spontaneous proteolysis, yielding a stable fragment with a molecular mass of ~18.5 kDa within several weeks (data not shown). A sample of this fragment was sequenced after reverse-phase high-pressure liquid chromatography purification (Applied Biosystems equipment; Edman degradation), and the following two sequences were observed: M A L K L E A D R L (major) and E R M A L K L E A D (minor).

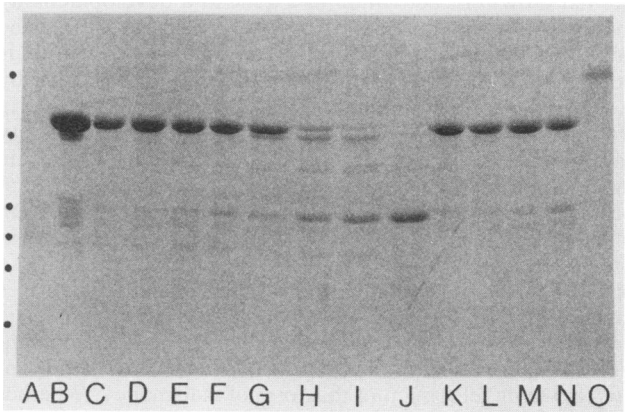

FIG. 1. Cleavage of Sindbis virus core protein as analyzed on a 15% discontinuous, denaturing polyacrylamide gel (11); time points are at 5, 15, 30, and 60 min. Lanes C through F show cores in 150 mM NaCl and 1:100 trypsin; lanes G through J show cores in 800 mM NaCl and trypsin; lanes K through N show cores in 150 mM NaCl, trypsin, and 1:100 RNase. Lane B was overloaded with intact cores, Sindbis virus glycoprotein was loaded onto lane O, and the dots at the left show the positions of standards at 43, 25.7, 18.4, 14.3, 6.2, and 3 kDa. Digestions were stopped with phenylmethylsulfonyl fluoride.

* Corresponding author.
† Present address: Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139.

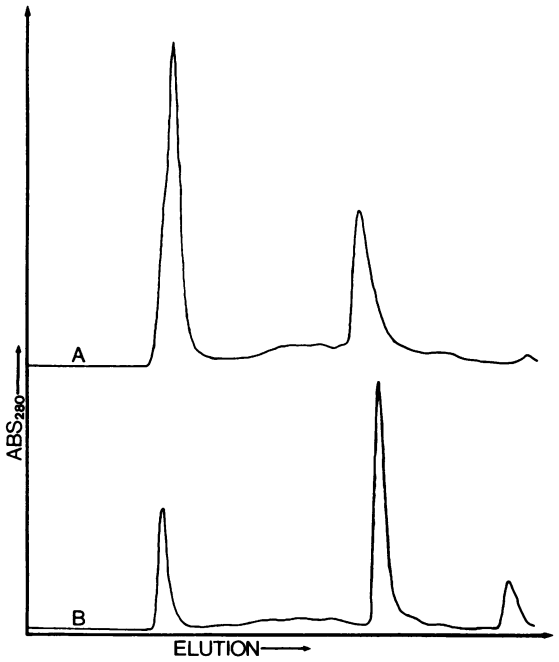

FIG. 2. Size exclusion chromatography of intact and cleaved core protein on Superose 12. Untreated cores were loaded and eluted in buffer containing 800 mM NaCl (dissociation of cores occurs readily at this salt concentration; trace A). Cores, dissociated in 800 mM NaCl and treated with trypsin as described in the text, were loaded and eluted in buffer containing 150 mM NaCl (trace B). The first peaks seen in each trace correspond to the void volume. ABS_{280}, Absorbance at 280 nm.

These two sequences correspond to proteolytic cleavages after Arg-103 and Arg-105 in the core protein sequence (12).

Trypsin cleavage of core protein. Figure 1 shows the result of attempting to reproduce trypsin cleavage of the core protein with Triton X-100-prepared cores. Incubation with trypsin in TNE at 4°C at a weight ratio of 1:100 (trypsin:core protein) did not yield significant amounts of the 18.5-kDa fragment, even after 4 h. However, in the presence of a salt concentration sufficient to dissociate the cores (800 mM), the intact core protein was readily digested to the 18.5-kDa fragment within 1 h at 4°C, without accumulation of any other fragment. This fragment was stable in the presence of 1:100 trypsin at 4°C for at least 4 h (data not shown). The primary sequence of this fragment as recovered from a single electroblotted band was M A L K L E and corresponds to a cleavage after residue 105 in the core protein sequence. The addition of 1:100 RNase A (Boehringer Mannheim Biochemicals) and 1:100 trypsin at 150 mM NaCl did not result in an increase in the cleavage of the core protein, despite the report that the core particle structure is sensitive to the addition of RNase (3).

Gel filtration of cleaved core protein. Figure 2 shows the result of gel filtration of the 18.5-kDa fragment and the intact core protein on a Pharmacia fast protein liquid chromatography Superose 12 column. Intact cores dissociated rapidly when loaded and run on a a column equilibrated with 800 mM NaCl. Two peaks were seen: a large peak at the void volume, consisting of the viral RNA and any trace glycoprotein present (shown by optical density at 260 nm/optical density at 280 nm ratios and gel electrophoresis of lyophilized column fractions; data not shown), and a peak containing only intact core protein at an elution volume corresponding to a molecular mass of 32.9 kDa. When dissociated, trypsin-treated cores were loaded and run on a column equilibrated with 150 mM NaCl, the peak corresponding to the cleaved core protein (as shown by gel electrophoresis; data not shown) eluted at a volume corresponding to a molecular mass of 20.8 kDa, very close to the expected monomer molecular mass of 18.5 kDa.

Conclusion. The observation that the C-terminal 18.5-kDa fragment is not cleaved at its potential trypsin sites in the presence of protease suggests that it is a compactly folded domain. Moreover, no species other than the 18.5-kDa fragment accumulates during digestion of the core protein with trypsin. We conclude that cleavage occurs at a large number of points in the N-terminal region and that these 103 residues are not folded into a stable structure at dissociating salt concentrations. However, in intact nucleocapsids, the N-terminal arm is insensitive to the addition of protease, suggesting that the structure of the core makes it inaccessible to protease, and the high proportion of basic residues indeed suggests strong interaction with viral RNA. This organization is similar to that seen in the coat proteins of turnip crinkle virus and tomato bushy stunt virus (8, 10). Experiments with turnip crinkle virus coat protein showed a similar pattern of protease sensitivity and lack of stably folded structure for the N-terminal arm (6, 9). In contrast to the coat protein of turnip crinkle virus and its stable fragment, which are dimeric in solution (6, 9), the 18.5-kDa fragment of the core protein, at 150 mM NaCl, and the intact core protein, at 800 mM NaCl, appear to be monomeric.

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