PREPARATION AND ANALYSIS OF CRYSTALLINE MACROMOLECULES

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Production and Crystallization of Virus-Like Particles Assembled in a Heterologous Protein Expression System

BY B. R. MCKINNEY, D. AGRAWAL, A. J. FISHER* AND J. E. JOHNSON†

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907, *USA*

AND A. SCHNEEMANN AND R. R. RUECKERT

Institute for Molecular Virology, University of Wisconsin, Madison, Wisconsin 53706, *USA*

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Abstract

It is of considerable interest to separate the processes of viral infectivity and virion assembly. Until recently this has only been possible with viruses that could be disassembled and reassembled *in vitro*. Even in these cases it was difficult to establish the authenticity of reassembled capsid protein because of possible irreversible damage that may have occurred to the protein during disassembly. An ideal method for the study of virus assembly is a protein expression system in which conditions are appropriate for spontaneous particle formation from freshly synthesized polypeptides. The baculovirus expression system has proven to be an excellent means to this end. Recently, this approach has been used to study the $T = 3$ Flock House insect virus and it has been demonstrated that subunits with the wild-type protein sequence, and with site-specific mutations that prevent particle maturation, will assemble and crystallize. This same approach has now been used at Purdue to study the $T = 4$ *Nudaurelia* ω *capensis* insect virus. There is no cell culture system currently available for the study of NωV, thus the expression system provides the first opportunity to study assembly under controlled conditions.

Abbreviations

ADA, *N*-(α-acetamido)-2-iminodiacetic acid, *N*-(carbamoylmethyl)iminodiacetic acid (from Sigma Chemicals). BME, β-mercaptoethanol. HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid. SF9, *Spodoptera frugiperda* ovarian cells. SF21, *Spodoptera frugiperda* ovarian cells – recommended for higher level production, larger in size than SF9. *T.ni*, *Trichoplusia ni* midgut cells. Hi5, *Trichoplusia ni* derived from egg-cell homogenates. FHV, Flock House virus. NωV, *Nudaurelia capensis* ω virus. sFHV, synthetic Flock House virus – virus-like particles with native FHV sequence whose proteins have been expressed in the baculovirus system. Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane. Tris–HCl, tris(hydroxymethyl)aminomethane hydrochloride. MOI, multiplicity of infection.

Introduction

The assembly of protein and nucleoprotein complexes is a common theme in biology and it requires a remarkable blend of regulation and complimentarity between the associating species. Simple icosahedral viruses are readily accessible for high-resolution studies by X-ray crystallography and their structures have had significant impact in understanding the principles of macromolecular assembly. To develop this understanding fully it is of interest to study virus particles that may not be infectious, composed of capsid proteins with specific mutations and it is desirable to have milligram quantities of these particles for crystallization. The baculovirus expression system has proven to be an excellent means to this end since, in many cases, freshly synthesized viral subunits spontaneously assemble to form virus-like particles in large quantity (*e.g.* Louden & Roy, 1991). We have used this approach to study Flock House virus (FHV) which contains 180 identical subunits, and we have shown that subunits with the wild-type protein sequence, and with site-specific mutations that prevent particle maturation and infectivity, will assemble and crystallize (Schneemann, Dasgupta, Johnson & Rueckert, 1993; Fisher, McKinney, Schneemann, Rueckert &

* Present address: The Enzyme Institute, University of Wisconsin, Madison, Wisconsin 53706, USA.

† Corresponding author.

Johnson, 1993). *Nudaurelia ω capensis* virus (NωV), which contains 240 identical subunits, also assembles in this expression system and this is the only means to study its assembly under controlled conditions, because there is no cell culture system available at present to support the growth of NωV.

A member of the *Nodaviridae* family, FHV is one of the simplest multipartite animal viruses. FHV maturation includes a post assembly cleavage of the capsid protein and this is necessary for infectivity and particle stability. The FHV genome consists of approximately 4500 bases that are split between two single-stranded messenger-sense RNA molecules encapsulated in one virion (Newman & Brown, 1973). The genome encodes only three proteins (Friesen & Rueckert, 1981), a replicase (protein A), the coat protein precursor (protein α which has 407 amino acids), and a protein of unknown function (protein B). The virus assembles to form a provirion with 180 identical capsid proteins assembled in a $T=3$ surface lattice (Caspar & Klug, 1962). The maturation cleavage occurs between Asn363 and Ala364 to produce peptide β and γ, which contains 363 and 44 amino acids, respectively (Hosur *et al.*, 1987). This cleavage, which stabilizes and enables infectivity, may be related to similar events in the maturation of picornaviruses (Hogle, Chow & Filman, 1985). The structure of the mature FHV is known at 2.8 Å resolution (Fisher & Johnson, 1993), but the structure of the provirion is not known because the maturation of the provirion to the virion has a half life of 4 h (Gallagher & Rueckert, 1988). The wild-type viral subunit sequence was expressed in the baculovirus system by inserting a cDNA clone of the FHV capsid protein in place of the polyhedrin gene. This resulted in large-scale production of viral subunits which assemble to form virus-like particles that package the mRNA of the capsid protein (Schneemann *et al.*, 1993). Mutations made at the cleavage site (Asn363/Ala364) prevent particle maturation leading to the formation of stable provirion-like particles in the expression system (Schneeman *et al.*, 1993).

In contrast to FHV, NωV is a $T=4$ virus. A member of the family *Tetraviridae*, NωV is a bipartite virus with two messenger-sense RNA's (Agrawal & Johnson, 1992). Each of the 240 subunits contains 645 amino acids and the subunits assemble into a provirion. As with FHV, an autocatalytic maturation cleavage occurs in this protein (α) between Asn570 and Phe571 to form β and γ (Agrawal & Johnson, 1992). NωV is found in the wild infecting Pine Emperor Moth larvae and it has not been grown in any cell-culture system; thus the role of cleavage with respect to infectivity has not been experimentally established as it has with FHV. As NωV is unable to be grown in a cell line (Moore, 1991), the expression system is the only dependable source of particles, as well as the means for studying assembly controlled conditions. At present only the native capsid protein has been expressed and this results in the formation of native virus-like particles. The expression was achieved by cloning the capsid protein coding sequences in *E. coli*. Anti-NωV serum (generated from mice innoculated with native virus) was used to verify the coding region in the primary translation product in the 70 kDa protein (Agrawal & Johnson, 1992). There was no cleavage of the precursor observed in protein expressed from *E. coli*. A recombinant baculovirus was created by inserting the cDNA, which encodes the capsid protein, into a transfer vector. The recombinant transfer vector was then co-transfected with the baculovirus DNA. Gene replacement resulted in NωV capsid protein under the control of a polyhedrin promoter and concomitant loss of polyhedrin synthesis. This recombinant 'virus' was then grown in SF9 and SF21 insect cell lines obtained from Invitrogen Corporation (San Diego, CA). Large-scale purification has not yet been attempted due to the relatively low yields of the particles.

Materials and methods

Purification

'Synthetic' FHV and its mutants have been successfully grown in SF9, SF21, *T.ni* and Hi5 cell lines also obtained from Invitrogen Corporation. Monolayered cells were infected with recombinant baculovirus inoculum using an MOI of 10 and were then allowed to grow for 3 d. The cells were removed and disrupted by adding 0.5% NP40 and 0.1% BME. Calcium chloride (5 m*M*) was added to all buffers during purification to increase the provirion stability (Gallagher & Rueckert, 1988). Cell debris was removed with low-speed centrifugation before precipitating the particles with 8% PEG 8000 and 0.2 *M* NaCl for at least 1 h. This suspension was centrifuged for 10 min at 14 000*g* to pellet the particles. The pellet was covered with 50 m*M* HEPES pH 7.0 with 5 m*M* $CaCl_2$ and allowed to resuspend at 277 K. A second low-speed spin was used to remove insoluble material and the virus-containing supernatant was then centrifuged in a 50.2 Ti Beckman rotor tube with a 30% sucrose cushion at 244 717*g* for 2 h at 277 K. The pellet was resuspended in 500 µl of the HEPES/$CaCl_2$ buffer before placing it on a 7.5–45% sucrose gradient. This was centrifuged in a Beckman SW41 Ti tube at 178 244*g* for 1.5 h at 277 K in a Beckman SW41 Ti swinging-bucket rotor. The viral band was removed by fractionation and centrifuged in a Beckman 50.2 Ti tube at 244 717*g* for 2 h at 277 K. An average of 1 mg of particles was obtained from 8×10^7 cells.

Crystallization

Both provirion-like particles and native 'synthetic' particles were crystallized by hanging-drop vapor diffusion (Fisher *et al.*, 1993). The first crystals of reasonable size and morphology were obtained with a solution of 5.0%(*w*/*v*) PEG 8000 (Fisher Scientific) in 10 m*M* sodium phosphate with 20 m*M* $CaCl_2$ at pH 6.8 in the reservoir and a drop prepared by mixing equal volumes (4–5 μl) of reservoir buffer with 'virus' solution at 12 mg ml^{-1} in 10m*M* Bis-Tris pH 6.0 (unless otherwise noted, all chemicals were obtained from Sigma Chemical Company). Crystals also appear when sodium phosphate is replaced with the following buffers, all at 10 m*M* concentration: ADA pH 6.5, HEPES pH 7.0, Tris–HCl pH 7.2 and Bis-Tris pH 6.0. This differs somewhat from the crystallization conditions of native FHV which were: 10 m*M* Bis-Tris pH 6.0, 2.8% PEG 8000 (Fisher Scientific), 20m*M* $CaCl_2$ and 0.001 *M* NaN_3 as a reservoir solution. This is combined in a 1:1 ratio with the virus solution of 18 mg ml^{-1} in 10 m*M* Tris–HCl pH 7.2 equal 20 μl drops which are grown using sitting-drop vapor diffusion at 293 K.

Results and discussion

Crystals with rhombohedral shape (Fig. 1) appeared in 4 weeks and diffracted X-rays from a synchrotron radiation source beyond 3.3 Å (0.33 nm) resolution (Fig. 2). Although the crystals appeared to have the same morphology as native FHV crystals, the provirion-like crystals did not have the same space group as native FHV, which was propagated in cultured *Drosophila melanogaster* cells. This difference may result from surface changes caused by the uncleaved capsid protein that could alter crystal packing or from growing baculoexpressed particles in a different cell line from native FHV (Fisher *et al.*, 1993). Native FHV crystallized in the trigonal space group *R*3 (Fisher, McKinney, Wery & Johnson, 1992) while the 'synthetic' particles crystallized in the monoclinic space group with cell parameters: $a = 464.8$, $b = 333.9$, $c = 325.2$ Å and $\beta = 91.9°$. The volume per molecular weight (V_m) (Matthews, 1968) implied two provirion-like paricles per unit cell with a value of 2.74 $Å^3$ Da^{-1}. This compares with 2.70 $Å^3$ Da^{-1} for the trigonal native FHV crystals indicating equivalent solvent content in the crystals (Fisher *et al.*, 1992).

Diffraction intensities were measured with the program developed by Rossmann (1979). An analysis of systematic reflection absences showed that the lattice is primitive. Examination of particle packing suggested that the space group is $P2_1$ with 300 Å particles at $(\frac{1}{4},y,\frac{1}{4})$; however, $0k0$ reflections were not present on the diffraction patterns analyzed (Fisher *et al.*, 1993).

The data were collected at the Cornell High Energy Synchrotron Source (CHESS). We would like to thank Sharon Fateley for her help in the preparation of this manuscript. This work was supported by a grant from the National Institutes of Health (GM34220) to JEJ.

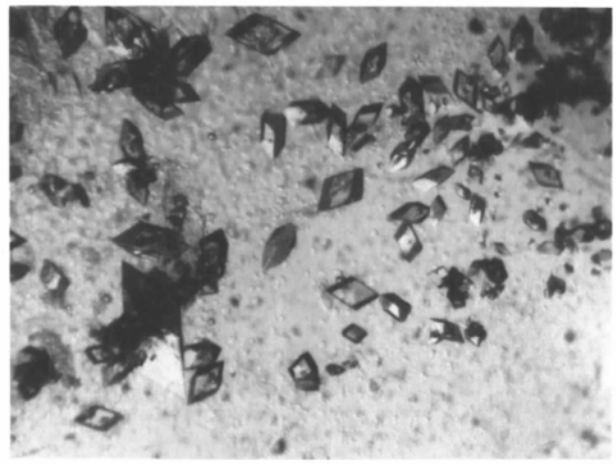

Fig. 1. Crystals of provirion-like particles assembled from mutant Flock House virus capsid protein expressed in a baculovirus system. The largest crystal is 0.3 mm across the body diagonal.

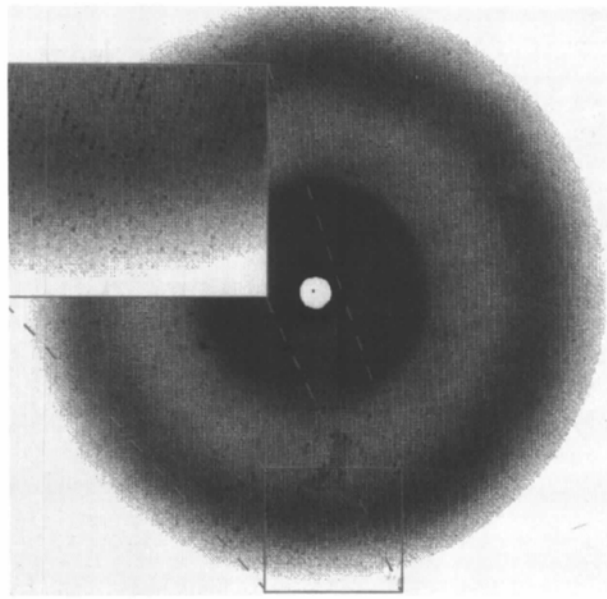

Fig. 2. A 0.5° oscillation diffraction pattern obtained from a crystal in Fig. 1, taken at the F1 line at the Cornell High Energy Synchrotron Source. The wavelength was 0.910 Å and the crystal-to-film distance was 220 mm. The resolution at the edge is 3.3 Å. A total of 10 727 reflections with $I/\sigma(I) \geq 2.0$ were accepted for processing on this film.

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