Electron Microscopy of Bacteriophage $\phi 6$ Nucleocapsid: Two-Dimensional Image Analysis

H. THOMAS STEELY, JR., AND DIMITRIJ LANG*

Molecular Biology Program, The University of Texas at Dallas, Richardson, Texas 75080

Received 22 November 1983/Accepted 23 March 1984

Electron micrographs of negatively stained nucleocapsids isolated from intact, wild-type $\phi 6$ bacteriophage revealed three distinct morphological forms. Two-dimensional analysis of electron micrographs of two of these forms and image averaging of all forms are consistent with a dodecahedral structure embodied in the $\phi 6$ nucleocapsid.

The *Pseudomonas phaseolicola* bacteriophage $\phi 6$ (18) is the only known bacteriophage having double-stranded RNA as the genomic agent (16). Its isometric nucleocapsid (NC) is enclosed in a phospholipid-protein envelope (6, 17). The NC can be separated from the envelope by nonionic detergent and remains structurally intact in the presence of divalent cations (17). Chemically, the NC consists of the four early proteins P1, P2, P4, and P7 plus one of the late proteins, P8, as well as three polycistronic double-stranded RNA segments, each of distinct length (1, 4).

The morphology of the NC and NC derivatives, as revealed by electron microscopy (EM), is clearly different from that of other known isometric virus particles. NCs isolated from mature $\phi 6$ virions are 45 to 50 nm in diameter and display a complex shape (2). From cells infected by either wild-type phage or nonsense mutants deficient in P8 synthesis, Mindich and Davidoff-Abelson (14) have isolated a particle which they propose to be a precursor of mature NCs. These particles (120S) contain the phage proteins P1, P2, P4, and P7 but not P8 and RNA. The authors (14) found that the 120S fraction contains three types of particles: stars, circles with 10 dots, and hexagonal structures. They have suggested that the star form results from a small number of substructural transitions in the 120S particle. Emori et al. (5) have found evidence of two nucleocapsid precursors (previrions I and II) which display morphologies similar to that of the 120S particle described above.

In this study, we isolated the NC from wild-type $\phi 6$ phage by using Triton X-100. NC preparations, negatively stained by phosphotungstic acid (PTA) for EM, confirmed the existence of three type of particles: (i) rings with 10 dots, (ii) irregular hexagons, and (iii) sixfold stars. Rotational averaging of images of these three forms showed five-, two-, and threefold axes of symmetry, respectively. Measurement of the geometric proportions of particle types (i) and (ii) showed that they were, within measurement error, identical to corresponding proportions of a regular dodecahedron, but different from those of a regular icosahedron. On this basis, we propose that the three morphological forms of the NC seen in this study by EM and the three types of 120S particles (14) are different views of a single structure, common to mature and immature NCs, and that this structure is dodecahedral, at least when stained by PTA.

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MATERIALS AND METHODS

Propagation of $\phi 6$ bacteriophage. *Pseudomonas phaseolicola* HB10Y (ATCC 21781) and bacteriophage $\phi 6$ (ATCC 21781-B1; Fig. 1a) were provided by J. L. Van Etten, University of Nebraska. The bacteria were grown at $24 \pm 2^\circ\text{C}$ in SSM medium and inoculated with phage $\phi 6$ as previously described (18). Precipitation of bacteriophage in the lysate was accomplished by addition of flaked polyethylene glycol 6000 and 0.5 M NaCl (19). Further isolation and purification of the phage proceeded as given by Vidaver et al. (18).

Isolation of $\phi 6$ NC. In a modification of the technique established by Bamford et al. (2), 2 units, as measured by optical density at 260 nm (OD_{260}), of $\phi 6$ phage were suspended in 5 ml of KM buffer (12.5 mM potassium phosphate-1 mM magnesium sulfate [pH 7.0]) and brought to 0.1% (vol/vol) in Triton X-100 (polyethylene glycol *p*-isooctylphenyl ether). The solution was gently mixed, allowed to stand for 5 min at 4°C , and then centrifuged (8 min, 60,000 rpm, Spinco SW-65 Ti rotor). The supernatant phospholipid-protein and detergent were discarded, the pellet was washed in 0.5 ml of KM buffer, and the washings were also discarded. The final pellet was allowed to resuspend in KM buffer to give an NC concentration of 0.6 to 1.0 OD_{260} U/ml.

EM. Intact $\phi 6$ phage or $\phi 6$ NC was applied to Pt specimen grids covered with carbon films (prepared by vacuum sublimation onto mica). After 5 min, excess liquid was removed with a wedge of Whatman no. 1 filter paper, and a drop of aqueous 2% PTA, neutralized with KOH, was applied to the still-wet grids. Alternatively, samples were mixed 1:1 with 2% PTA (pH 7) and then deposited onto grids. Excess liquid was removed almost immediately with filter paper. Electron micrographs were made with a Siemens Elmiskop IA at 60 or 80 kV on Kodak electron image plates at electron optical magnifications of 2×10^4 to 8×10^4 .

Rotational averaging of NC images. The signal-to-noise ratio of selected images, and thus the recognition of rotational symmetry of the three morphological types of NCs, was improved by rotational photographic averaging (12).

Measurement of particle proportions. Geometric proportions within two of the morphological forms, (i) rings with 10 dots and (ii) irregular hexagons, were measured on photographic prints of the original electron image plates by using a calibrated ruler with 0.5-mm divisions. The NC diameter on prints was about 15 mm. There was no significant difference between proportions in free and clustered NCs.

Recovery of double-stranded RNA from $\phi 6$ NC. Purified NCs were disrupted with sodium dodecyl sulfate and sub-

* Corresponding author.

FIG. 1. Electron micrograph of intact (a) and partially disrupted (b) $\phi 6$ bacteriophages negatively stained with 2% PTA. The bar represents 100 nm.

jected to double-phase phenol extraction with 100 mM sodium phosphate buffer (pH 7.0). After extraction, the Na^+ was lowered to 1 mM by dialysis in the presence of 0.1 mM disodium EDTA. Thermal denaturation of the recovered nucleic acid was monitored at 258 nm with a Gilford 2000 recording spectrometer. The sample chamber was heated at a constant rate of $0.2^\circ\text{C min}^{-1}$ by a circulating ethylene glycol-water solution.

Zone sedimentation of $\phi 6$ NC. A suspension of purified NCs (2 OD_{260} units) was layered on 11 ml, 10 to 35% linear sucrose density gradients containing KM buffer and centrifuged (23,500 rpm, 90 min) in a Spinco SW41 rotor. After centrifugation, vertical illumination of the centrifuge tubes revealed only one light-scattering band near the center of each tube. The gradient was then partitioned into 0.25-ml fractions. Absorbance measurements of each fraction at 280 and 258 nm confirmed the presence of a single band. Fractions including the leading and trailing edges of the band were then examined by EM.

RESULTS

EM of $\phi 6$ and $\phi 6$ NC. Bacteriophage $\phi 6$, negatively stained by PTA (pH 7), showed little internal detail (Fig. 1a), but partial disruption of the envelope, during storage at 4°C before being mounted on grids, revealed both ring and hexagonal forms (Fig. 1b). After removal of the envelope, three distinct forms were persistently seen (Fig. 2a), descriptively identified as (i) irregular hexagons, (ii) rings (often with 10 dots), and (iii) sixfold stars. The four long edges of

the negatively stained, irregular hexagons were consistently seen in higher contrast than the two remaining edges. Forms (i) and (ii) were found with high frequency, and form (iii) was found with low frequency. Our sixfold stars differ from the stars, identified earlier (14), which have no symmetry axis.

Rotational image averaging. Figure 2b to d shows multiple exposures of NC images on photographic paper at n rotations of $2\pi/n$, in which n is an integer generating the best structural definition (12). Not shown are other n -fold rotations resulting in blurred images. We concluded that each of the three distinct forms has an axis of symmetry perpendicular to the plane of the image corresponding to these NC symmetry axes: fivefold for the ring, twofold for the irregular hexagon, and threefold for the sixfold star.

Zone sedimentation of NC. The question arose whether the three observed forms represented three different NC species. We therefore attempted to separate and isolate them by zonal sedimentation but were unsuccessful. Hence, the NC samples were either homogeneous or contained a mixture of particles whose differences in density and shape combined in such a way as to result in identical sedimentation velocities. EM examination of NCs from the leading and trailing edges of the single band, and from its center fraction, showed a uniform frequency ratio of hexagonal to ring forms of ca. 2:1.

Geometry of $\phi 6$ NC. The fact that orthogonal axes of two-, three-, and fivefold symmetry define icosahedral symmetry (3) led us to compare the NC images with the two simplest bodies of icosahedral symmetry: the regular icosahedron and the regular dodecahedron. Parallel projections of the edges

of these bodies, viewed along symmetry axes, are shown in Fig. 3. A projection along the twofold symmetry axis was strikingly similar to the irregular hexagon seen in Fig. 2b. This comparison is justified by the fact that electron micrographs are also parallel projections of the specimen since the numerical aperture of the objective lens is about 10^{-2} and $\ll 1$. Similarly, inspection of Fig. 3 and of the ringlike forms (e.g., Fig. 2a and d) also suggested a dodecahedron, but not

FIG. 2. Negatively stained (2% PTA) $\phi 6$ NCs. (a) Three distinct forms: irregular hexagons, star (inset), and rings with 10 dots. Three different forms from (a) were enlarged by the same factor and photographically averaged (12); (b) irregular hexagon, rotated twice about twofold symmetry axis; (c) star, rotated 6 times about threefold axis; and (d) ring, rotated 10 times about fivefold axis. Magnification reference: outer diameter of the ring forms is 46 nm.

FIG. 3. Parallel projections of the edges along the two-, five-, and threefold axes of symmetry for the dodecahedron (a to c) and the icosahedron (d to f). If these bodies were virus particles on a specimen grid in the electron microscope, they would be resting on a single edge (a and d), on a pentagonal (b) or triangular face (f), or on a vertex (c and e).

an icosahedron. Measurements of the dimensions of the two most frequent morphological forms, given as ratios (Fig. 4), supported this observation quantitatively.

Recovery of double-stranded RNA from $\phi 6$ NC. After phenol extraction of purified NCs, we observed peak UV absorption at 258 nm, an OD_{260}/OD_{280} ratio of 2.18, and, in the presence of 1 mM sodium phosphate buffer and 0.1 mM disodium EDTA, a thermally induced hyperchromicity of 35%, clearly indicative of double-stranded nucleic acid. The presence of double-stranded RNA in purified NCs shows that our NC samples and the 120S particles (14) differ chemically, although the 120S particles and mature NCs appear to have identical morphology when stained with PTA at pH 7.

DISCUSSION

We infer that the $\phi 6$ NC contains a structure which has icosahedral symmetry and can best be described as a regular dodecahedron and that the three remarkably different forms determined by EM after PTA negative staining are merely different views of that structure. If this is true, the $\phi 6$ NC would be the first known example of a virus-related particle having such an architecture. Evidence in favor of this inference is as follows.

(i) The failure of our attempts to separate the three distinct morphological forms by zonal sedimentation suggests that there is only one species of NC. Support for this inference is presented in the accompanying paper (20).

(ii) Each of the three forms observed has its own symmetry axis (Fig. 2) corresponding to NC axes of symmetry. If there is indeed only one NC species, the NC must then have two-, three-, and fivefold axes of symmetry and hence, icosahedral symmetry. This would satisfy the constraints on the geometry of an isometric virus as discussed by Caspar and Klug (3, 11) and Klug (10).

The surface/volume ratio of a regular dodecahedron is only 3.2% higher than that of a regular icosahedron. Thus, containment of the genome in a dodecahedral framework should not be a significant disadvantage in this regard.

(iii) Electron micrographs are parallel projections of the NC particles and therefore directly comparable with parallel projections of model structures. The simplest bodies of

FIG. 4. Geometric proportions of the hexagonal and the ring form seen in nucleocapsid preparations in comparison with the corresponding proportions for a regular dodecahedron and icosahedron. Measured were 19 hexagonal and 22 circular particles. The u/v and x/y ratios, respectively, were as follows: observed, 1.16 ± 0.04 and 1.86 ± 0.13 (the errors are sample standard deviations); for a dodecahedron, 1.176 and 1.902; for an icosahedron, 1.070 and 1.236.

icosahedral symmetry are the regular icosahedron and the regular dodecahedron, having parallel projections as illustrated in Fig. 3. We have shown (Fig. 4) that the geometric proportions in the NC images of rings and irregular hexagons are, within measurement error, those of projected regular dodecahedra and not of regular icosahedra. Among the more complex bodies of icosahedral symmetry is the deltahedral family of polyhedra (3) whose faces are equilateral triangles ("icosadeltahedra"). An example is the pentakis dodecahedron (9), illustrated in reference 13. We did not find evidence that would favor an icosadeltahedron over a simple, regular dodecahedron.

(iv) Parallel projection along the threefold axis of symmetry implies that the NC rests on a vertex if it is a dodecahedron and on a face if it is an icosahedron (Fig. 3). The rarity of star forms in our samples, together with the assumption that an NC is unlikely to rest on a vertex (as yet unproven), would suggest that the NC has a dodecahedral rather than icosahedral surface.

The question must be raised as to whether or not the observed structures are preparative artifacts. We are unaware of proof that negative staining with PTA generates virus structures that are absent without PTA. However, it has been shown that PTA reveals structures in vaccinia virus selectively, depending on the pH (15), and that this selectivity is reversible (8). We therefore assume that the structures in our micrographs of NCs negatively stained with neutralized PTA are real but that the NC may contain (besides RNA) other structural elements.

Deformation of NCs in PTA during drying may pose another problem. NCs dried from suspensions without PTA and shadowed with platinum (not shown) were significantly flattened, presumably by surface forces at the time when the solution-air interface passed through the NCs. However, one may expect that flattening is reduced in the presence of PTA, when the glassy PTA precipitate forms a supporting matrix around the NCs, before the interface has passed them. Indeed, tilting the specimen grids has demonstrated (20) that the spatial structure is sufficiently preserved to permit a three-dimensional analysis.

Elimination of structures by PTA is another possibility. Using uranyl acetate as a negative stain, the morphologies of $\phi 6$ NCs differ from those obtained by PTA (reference 14 and our unpublished data). Mindich and Davidoff-Abelson (14) point out that $\phi 6$ NCs stained with uranyl acetate appear to have an outer layer not observed with PTA and suggest that PTA may remove the outer layer containing the protein P8. Unfortunately, the geometric structures discussed above are in general not clearly delineated in mature NCs stained with uranyl acetate so that it is difficult

to view the isometric structure and the outer layer simultaneously. However, Fig. 4d of reference 14 does show a uranyl-stained 120S particle in star form, indicating that this form is either real or that both stains induce the same artifact. The only proof of structural reality when employing EM is indirect: by demonstrating that many different preparation methods produce consistent results. It remains to be seen in this case whether or not our proposed NC structure can be confirmed by other techniques such as cryofixation with carbon replication and double-angle shadowing.

We have not observed irradiation damage of the morphological features as described; these appear to remain stable in the electron beam after the first viewing.

The structures in our NC micrographs were not induced by Triton X-100 since $\phi 6$ preparations, partially disrupted by storage at 4°C but not treated with the detergent (Fig. 1b), showed ring, hexagonal and star forms indistinguishable from those of isolated NCs. Also, the same three forms are seen among *sus297* NC particles isolated in the absence of Triton X-100 (14).

It is presently not clear how an icosahedral NC structure or the proposed dodecahedral structure could be reconciled with the copy number of protein components per NC (4) and the Caspar-Klug theory (3) of capsid structure. An intriguing possibility consistent with our data and supported by Fig. 4 in reference 20 is that the structure is a rather open dodecahedral cage whose edges are assembled from rodlike proteins. An example of such a dodecahedral protein cage is seen in the multienzymic complexes of pyruvate dehydrogenase isolated from *Bacillus stearothermophilus* (7). Depending on their orientation on the specimen grid, these complexes clearly displayed three forms in the EM similar to those in Fig. 3a, b, and c.

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