Studies on the Nucleocapsid Structure of a Group A Arbovirus

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When Sindbis virus (273S) was treated with sodium desoxycholate, a nonhemagglutinating 136S particle was liberated from the virion, representing the viral nucleocapsid (core). Electron microscopically it appeared as a spherical particle 35 nm in diameter, showing ringlike morphological units 12 to 14 nm in diameter on its surface. When the one- and two-sided images of core particles were correlated, their structure could be demonstrated to have the $T = 3$ arrangement of 32 hexamer-pentamer morphological units within a symmetrical surface lattice. The core contained a further spherical structure (12 to 16 nm in diameter) which was designated as the central core component. Two proteins were found associated with the core, a third viral protein belonged to the hemagglutinating surface structures. The significance of these findings for virus classification is discussed.

Arboviruses of the serological group A are known to be lipid-containing ribonucleic acid (RNA) viruses (22, 16) which consist of a core particle enveloped by a membrane (1, 10, 12, 13, 15, 18). No direct evidence for the structure and symmetry of the core was so far obtained, apparently because the viral envelope interfered with direct observation of the nucleocapsid in intact particles. After chemical treatment, however, regular patterns emerged in isolated viria suggesting a cubic core symmetry (18, 14).

The present paper reports studies on the nucleocapsid structure and symmetry after *in vitro* decoating of the virus particle.

MATERIALS AND METHODS

Virus. A mouse-adapted Sindbis virus strain was used. This strain (AR 86; passage 63) was originally obtained from B. M. McIntosh (The South African Institute for Medical Research, Johannesburg, South Africa). All experiments reported here were performed with material from the 14th passage of the strain in chick embryo cells.

Assay methods. Infectivity was titrated using the plaque technique in chick embryo cells as described previously (12). Hemagglutinating activity was tested by a micromethod (17), in which the reaction mixture was adjusted to pH 6.0 (4).

Virus purification. The methods of preparation of chick-embryo cell monolayers and of high titered pools of Sindbis virus have been described in detail (12). Infectious tissue culture fluids were harvested after 18 hr of incubation at 37°C and clarified by centrifugation at $2,200 \times g$ for 30 min. The virus was allowed to adsorb onto freshly prepared AlPO_4 gel equilibrated with

a 0.05 M phosphate buffer (pH 8.0). It was eluted from the gel in 0.3 M phosphate buffer (16) and was concentrated by centrifugation at $78,000 \times g$ for 60 min. The pellet was resuspended in a buffer solution (Tris-saline) containing 0.02 M tris(hydroxymethyl)aminomethane (Tris), 5×10^{-4} M ethylenediaminetetraacetate (EDTA), and 0.1 M NaCl, adjusted to pH 8.5 by addition of HCl. Further purification was achieved by zonal sedimentation of the material through a linear sucrose gradient. Material of the opalescent band was collected, diluted in Tris-saline, and pelleted again ($105,000 \times g$ for 30 min). The resuspended sediment represented the starting material for all subsequent experiments.

Sucrose density gradient. The sample (2.0 ml) was layered on top of a 15 to 30% (w/w) sucrose gradient in Tris-saline in a lusteroid cellulose tube fitting the 25.1 rotor of the Spinco model L ultracentrifuge and was spun for 3 hr at 25,000 rev/min and 4°C. Fractions were collected through a hole in the bottom of the tube and optical densities at 254 nm were measured using a Unicam SP 700 Recording Spectrophotometer equipped with a flow cell.

Analytical ultracentrifugation. The sedimentation properties of Sindbis virus and its core particle were studied using a Spinco model E analytical ultracentrifuge equipped with a schlieren optical system. Virus preparations were standardized at an E_{254} of 3.0 and centrifuged at 20,410 rev/min and 8.5°C using 12-mm cells with a 4° sector (Kel-F centerpiece). Exposures were made at 4-min intervals. Observed sedimentation rates were converted to sedimentation coefficients by application of a desk top computer program (PI 1032) for the Olivetti Programma 101, kindly provided by R. Trautman (Plum Island Animal Disease Laboratory). Viscosities were determined using a capillary viscosimeter, type Ubbelohde (Schott u. Gen., Mainz, Germany).

Electron microscopy. Equal amounts of the specimen and a saturated aqueous solution of uranyl acetate were mixed and incubated for 2 min at room temperature before electron microscopy. For spray droplet preparations, samples of 0.1 ml were mixed with an equal quantity of an aqueous 2% solution of sodium phosphotungstate (PTA) at pH 6.0 and with 0.05 ml of glycerol. The preparations were applied to carbon films carried by formvar-coated copper grids; they were examined in a Siemens Elmiskop I ($\times 20,000$). The magnification was standardized using pictures of a carbon grating of known mesh size. Particle diameters were determined from electron micrographs (final magnification, $\times 180,000$) using a measuring lens.

Polyacrylamide gel electrophoresis. Samples were incubated for 60 min at 37 C in presence of 8 M urea and were incorporated into a 2.5% acrylamide gel. Electrophoresis was performed in glass tubes (6 by 120 mm) on vertical 80-mm gel columns composed of 10% acrylamide, 0.20% *N,N'*-bis-methylene acrylamide and 0.5 M urea in a 0.01 M Tris-glycine buffer (pH 8.6). Polymerization was catalyzed by final concentrations of 0.075% (v/v) *N,N,N',N'*-tetramethylethylenediamine and 0.06% ammonium persulfate. The buffer used in the electrode vessels was 0.01 M Tris-glycine (pH 8.6), containing 0.5 M urea. The samples were run at 3 v/cm for about 3 hr at room temperature. Gels were fixed and stained with 0.6% amido black in 7.5% acetic acid.

RESULTS

Preparation of Sindbis virus cores. Purified Sindbis virus was spun in the analytical ultracentrifuge in presence of 0.2% sodium desoxycholate (DOC), including a control in a wedge window cell. A slower component is released from the virion whose sedimentation coefficient was calculated at $136.2 \pm 1.0S$ as compared with $273.4 \pm 0.2S$ for the untreated particle (Fig. 1). The 136S component will subsequently be called "core."

To adapt this procedure to a preparative scale, Sindbis virus was layered on top of a sucrose gradient and centrifuged after treatment with 0.2% DOC in Tris-saline for 20 min at 4 C. This condition had been found optimal with respect to dissociation of core and membrane as reflected by reduction of virus infectivity (Fig. 2). For complete separation of hemagglutinating activity and extinction at 254 nm it was necessary that the gradient itself contained 0.2% DOC (Fig. 3). When the substance was absent, additional light scattering bands were observed near the bottom of the tube. They corresponded to partially degraded virus and particle aggregates, respectively, as evidenced by electron microscopy.

Size and shape of the viral core. Spray droplet preparations of Sindbis virus cores stained with PTA contained roughly spherical particles (Fig.

4) with an average diameter of 35 nm. No pentagonal or hexagonal outline could be discerned in them. Particles near the margin of PTA droplets were greater in diameter than those distant from it (43 nm). Correlograms have revealed an asymmetric deformation of the marginal core particles.

When stained with uranyl acetate and subsequently contrasted with PTA, the core had an average diameter of 35 nm. Cores that stained positively with uranyl acetate alone showed significantly smaller diameters (29 nm) than those obtained with negative staining methods. All measurements are summarized in Table 1; the average diameter of intact Sindbis virus stained with uranyl acetate is given for comparison (61 nm). The variability of the individual observations around their respective mean values was expressed in terms of the standard deviation. Furthermore, the coefficients of variation (standard deviation/mean value) are given in per cent values as a more compact measure of variability.

Substructure of the viral core. In preparations negatively stained with PTA, the cores showed a granular surface structure (Fig. 4). However, no

FIG. 1. Schlieren patterns at 20,410 rev/min of Sindbis virus and its core particle, liberated in presence of 0.2% DOC. Peaks correspond to 273S and 136S, respectively. Exposure was made 21.6 min after starting the run. Sedimentation is from right to left.

FIG. 2. Decrease of Sindbis virus infectivity after incubation with 0.2% DOC at 4 C for various periods of time.

evidence for the type of arrangement of morphological subunits could be obtained from these pictures. Staining with uranyl acetate was able to resolve ringlike morphological units 12 to 14 nm across in one-sided images of the core (Fig. 5). Their sterical arrangement could be demonstrated in particles additionally contrasted with PTA. The core surface is composed of regular pentagonal and hexagonal rings arranged on the surface

of a sphere (Fig. 6). The idealized distribution of intensity seen in a micrograph of a core particle viewed close to a fivefold axis of symmetry is presented in the corresponding drawing. The positions of the vertices of the polygons show as coordinates in an icosahedral surface lattice with a triangulation number of $T = 3$.

The expected electron density distribution in two-sided particle images was studied using shadowgraphs of a skeletal model constructed from 12 pentagons and 20 hexagons. Figure 7 shows good agreement between three representative two-sided images of the Sindbis virus core particle and the corresponding superposition patterns produced by the model.

Detection of a central core component. When core preparations were prefixed with osmium

FIG. 3. Rate zonal centrifugation of purified, DOC-treated (0.2%, 20 min at 4 C) Sindbis virus in a 15 to 30% sucrose gradient containing 0.2% DOC. Note complete dissociation of hemagglutinating activity (dashed line) and absorbance at 254 nm (solid line).

FIG. 4. Core particles of Sindbis virus, stained with PTA, pH 6.0 (spray droplet preparation).

TABLE 1. *Measurements from electron micrographs of Sindbis virus, its core and its central core component under different staining conditions*

Object	Staining method	Number of particles measured	Mean value (nm)	Standard deviation (nm)	Coefficient of variation
Intact virus.....	uranyl acetate (negative staining)	237	61.1	1.6	% 2.7
Core.....	uranyl acetate (positive staining)	300	28.8	1.5	5.1
	PTA (spray droplet)	107	34.9	0.4	1.1
	droplet center	324	43.3	0.4	0.9
	droplet margin				
	uranyl acetate and PTA (negative staining)	600	35.5	0.4	1.1
Central core component.....	osmium tetroxide and PTA	37	15.9	2.3	14.5
	uranyl acetate	32	11.8	1.2	10.2

FIG. 5. Core particle stained with uranyl acetate. Ringlike morphological units 12 to 14 nm in diameter are evident.

tetroxide vapor on the grid, subsequent staining with PTA revealed less dense and occasionally disrupted cores accompanied by spherical particles about 16 nm in diameter (Fig. 8a). In preparations of core material stained with uranyl acetate for about 5 min, similar particles (about 12 nm in diameter) were observed, forming a central structure within the intact core that appears surrounded by a narrow space (about 3 nm) of high electron density (Fig. 8b).

Proteins of the viral core. When cores prepared by DOC treatment of Sindbis virus and purified by zonal centrifugation in sucrose gradients in presence (Fig. 9c) or absence (Fig. 9d) of DOC were subjected to polyacrylamide gel electrophoresis, the two bands II and III were consistently observed. Corresponding bands were found in electropherograms of whole Sindbis

virus (Fig. 9a); the additional band (I) was present only in those fractions of the density gradient (Fig. 3) that showed hemagglutinating activity. Figure 9b demonstrates that it was not resolved under the experimental conditions employed.

DISCUSSION

The Sindbis virion is a roughly spherical particle (60 to 70 nm in diameter) that consists of an envelope bearing projections and a spherical core (12). Morphologically it resembles other arboviruses of the serological group A (13, 15,

FIG. 6. Core particles stained with uranyl acetate and then by PTA, pH 6.0. View is close to a fivefold axis. The corresponding drawing shows the idealized distribution of electron density and the correlation of morphological units with a $T = 3$ surface lattice (dotted lines).

FIG. 7. Sets of two-sided images of core particles from a spray droplet preparation with PTA, pH 6.0. The particle architecture closely resembles shadowgraphs of a model of 20 hexagonal and 12 morphological structural units arranged on the surface of a sphere (6).

FIG. 8. Demonstration of a central core component in Sindbis virus core preparations negatively stained (a) with PTA after fixation with osmium tetroxide and (b) with uranyl acetate.

18, 19). In our study, conditions were sought which would release the lipoprotein coat while leaving the nucleocapsid intact. Such a result was achieved by treatment of Sindbis virus with 0.2% DOC (20) and then by separation of the products in a sucrose gradient. Treatment with

DOC does not impair the hemagglutinating activity of the membrane structures, which can be recovered near the top of the gradient (Fig. 3). The sedimentation coefficient of 136S obtained for the core using the analytical ultracentrifuge compares well with the data (140S and 130S,

FIG. 9. Polyacrylamide gel electrophoresis of (a) whole Sindbis virus, (b) hemagglutinating material from density gradient fractions (see Fig. 3) and core material from gradients with (c) and without (d) DOC. The fast migrating bands in (b) and (c) represent the detergent.

respectively) from sucrose gradient runs of viral cores (20) and of intracellular precursor particles (2) of Sindbis virus.

Simpson and Hauser (18) have presented electron micrographs of arbovirus particles suggesting cubic symmetry. Pictures of whole Sindbis virus from suspensions pretreated with formaldehyde exhibited a sixfold radial symmetry due to a regular arrangement of spherical units, about 12.5 nm in diameter. Osterrieth (14), too, has demonstrated symmetrical core images of Semliki Forest virus with polygons 12 nm across on their surface; the virus had been decoated by the action of caseinase C at low salt concentration (15). On Sindbis virus nucleocapsids decoated by DOC treatment, we have found "hol-

low" polygonal morphological units 12 to 14 nm in diameter (Fig. 5 and 6) when using uranyl acetate as a negative stain; its advantages over PTA have been discussed by Finch and Holmes (5).

As pointed out by Caspar and Klug (3), an isometrical particle is most likely to have icosahedral symmetry if at least one 5-coordinated morphological unit is seen (Fig. 6). From the small number of units discernible on the core surface, only the lower members of the icosahedral classes $P = 1$ (42 units) and $P = 3$ (32 units) can be considered. In the Sindbis virus core particle, the $P = 1$ class was excluded because of the following observations. (i) No case where a pair of 5-coordinated units is seen on either side of a single 6-coordinated unit was distinguished, as would be expected if the number of morphological units were 42 (3). Instead, 5-coordinated units were found surrounded by 5 hexagonal neighbors (Fig. 6). (ii) The surface occupied by 32 units 12 to 14 nm across can cover a sphere of about 37 nm in diameter; the diameter of a sphere with 42 units of the same size on its surface would be more than 10% greater than the values measured, which is beyond the coefficients of variation (Table 1). (iii) Favorably oriented two-sided images of core particles showed an electron density distribution comparable with shadowgraphs of a 32-unit model similar to that constructed for turnip yellow mosaic virus (6). Since these pictures do not show a great deal of substructure, they cannot be considered as proving the type of symmetry suggested; they are consistent with the data, however. Consequently, the morphological subunits on the Sindbis virus nucleocapsid are arranged within a $T = 3$ surface lattice. Clustering to solid capsomeres does not occur under normal conditions which explains the particle images of a circular rather than a polygonal silhouette. Treatment with formaldehyde (18) or heat (M. Horzinek, unpublished data) occasionally produces images of particles showing distinct capsomeres.

Negative staining has revealed a central core component 12 to 16 nm in diameter. In ultrathin sections through Middleburg virus particles, another group A arbovirus, Simpson and Hauser (19) have observed a "central body" 8.5 to 12.5 nm across within the core, which was not detected in Sindbis viria. A "dark central spot" was occasionally seen in thin sectioned Semliki Forest nucleoids (1). It remains to be shown whether the central core component is common to group A arboviruses.

Strauss et al. (20) attributed one major protein to the Sindbis core. Our results support the findings of Friedman (7) who detected two core pro-

teins in another group A arbovirus (Semliki Forest virus). The discrepancy between our results and those obtained by Strauss et al. (20) may be due to the different techniques employed. The authors were working with labeled proteins, whereas, in our experiments, bands were localized by direct staining. According to Strauss et al. (20), a polypeptide occurring only once or twice per virion would not be found; when poorly labeled, our minor core protein may escape detection (Fig. 9, band II). It was also present when sodium dodecyl sulfate-urea-mercaptoethanol-treated samples (21) were analyzed (M. Horzinek, unpublished data). Current studies try to correlate the two core polypeptides with the central core component and its capsid shell.

We have shown that a member of the serological group A of arboviruses possesses a nucleocapsid with icosahedral symmetry. Since antigenically related viruses share some structural properties (11), this most probably holds true for the whole group. With one exception (8), most workers agree with this concept, which is based on electron microscopical observation of virus particles (11, 14, 18) and of paracrystalline arrays of virus nucleoids in the cytoplasm of infected cells (1).

Viruses with the architecture of Sindbis virus must be considered as the "missing link" in the system of viruses developed by Lwoff, Horne, and Tournier (9), filling the gap of enveloped RNA viruses with cubic symmetry of the nucleocapsid. The jargon name "togaviruses" has been suggested for this new structural class by the Arbovirus Study Group. It would comprise other viruses of a similar architecture that are not transmitted by arthropod vectors (possibly rubella, hog cholera, bovine viral diarrhea viruses, etc.).

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