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Quantitative dissociation of archaeal virus SH1 reveals distinct capsid proteins and a lipid core

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Abstract

Viruses infecting archaeal cells are less well understood than those infecting eukaryotic and bacterial cells. Here we study the distribution of the structural proteins between the capsid and the membrane of icosahedral SH1 virus, an archaeal virus infecting extreme halophilic *Haloarcula hispanica* cells. General features such as morphology, linear dsDNA genome and presence of lipids suggest that it may belong to the recently proposed PRD1-adenovirus lineage of viruses. To investigate this we have initiated structural studies of the virion. Quantitative dissociation of SH1 by 3 M urea or by lowering the salt concentration identified a number of soluble capsid-associated proteins (VP2, VP3, VP4, VP6, VP7 and VP9). These released proteins left behind a particle, or lipid core, containing two major proteins VP10 and VP12 and viral phospholipids. VP1 was released from the lipid core in low ionic strength conditions but not with 3 M urea. Approximately half of the protein VP5 stayed with the lipid core and the other half was released. Analysis of the soluble capsid-associated proteins by their sedimentation and hydrodynamic properties suggests that the most abundant proteins, putative capsomers VP4 and VP7, form an intricate pattern of protein complexes. We also observed large differences in the sizes of the complexes determined by the two different methods suggesting an elongated overall structure for most of the capsid-associated proteins or protein complexes. This work verifies that there is an internal membrane vesicle residing inside the complex icosahedral capsid that is akin to the overall structure of PRD1-like viruses.

Keywords: Archaea; Archaeal virus; Halobacteria; Haloarchaea; *Haloarcula hispanica*

Introduction

Isolated, and to some extent characterized, archaeal viruses represent a small minority among the more than 5000 different prokaryotic viruses described to date. There are more than 20 viruses reported to infect euryarchaeal extreme halophiles and methanogens and some 25 viral species that infect thermophilic Archaea belonging to the crenarchaeal kingdom (Stedman et al., 2006). The majority of the viruses infecting euryarchaeal cells are similar to the most common class of bacteriophages, i.e., head–tail viruses with dsDNA genomes (Ackermann, 2003; Dyall-Smith et al., 2003). Viruses infecting members of the *Crenarchaeota*, on the other hand, differ considerably from the tailed bacteriophage morphology. For these viruses, the new families *Lipothrixviridae*, *Rudiviridae* and *Guttaviridae* have been established. In addition, at least three more families have been suggested on the basis of new morphologies (Prangishvili and Garrett, 2005). The family *Fuselloviridae* contains lemon-shaped viruses that infect crenarchaeal (genus *Fusellovirus*) hosts. Lemon-shaped viruses infecting euryarchaeal cells belong to genus *Salterprovirus*. Another feature that separates the euryarchaeal and crenarchaeal viruses is their virulence. Euryarchaeal viruses are often virulent whereas the crenarchaeal ones are temperate with only few exceptions. Examination of samples taken directly from hypersaline environments

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(Oren et al., 1997) or from enrichment cultures of thermophilic archaea (Rachel et al., 2002; Rice et al., 2001) demonstrates that only a minority of archaeal virus types have been thus far isolated.

SH1 is a recently identified lytic virus that infects the extremely halophilic euryarchaeal species *Haloarcula hispanica* (Porter et al., 2005). Icosahedral SH1, which is approximately 70 nm in diameter, possesses a 31-kb linear double-stranded DNA genome that encodes 56 predicted open reading frames (Bamford et al., 2005b). The genome has 309-bp inverted terminal repeats. SH1 has approximately 15 structural protein species of which 11 have been identified either by N-terminal sequence analysis and/or by mass spectrometry. Most of the genes encoding the identified structural proteins reside in the middle of the genome. The primary amino acid sequences of most of these proteins do not share any significant sequence similarity with any other sequence in the databases (Bamford et al., 2005b), except for ORF 17 that shares homology with viral packaging ATPases (Strömsten et al., 2005). In addition, the 81-kDa VP2 has been suggested to have an elongated structure consisting of different domains and resembling fiber-like proteins often involved in forming viral spike structures (Bamford et al., 2005b; Caldentey et al., 2000; Merckel et al., 2005). Another structural protein in the SH1 virion is the 152-kDa VP1, which has been shown to form homomultimeric complexes (Bamford et al., 2005b). The VP4 (25 kDa) and VP7 (20 kDa) proteins are candidates to form the viral capsid. These proteins have been shown to form homo- and heteromultimeric complexes in the size range of 45 to 75 kDa. The virion also contains lipids selectively derived from the host and most probably situated underneath the protein capsid (Bamford et al., 2005b).

Although SH1 proteins share very little homology with proteins in the databases, common features such as icosahedral morphology, linear dsDNA genome and presence of lipids suggest that SH1 may belong to the lineage of icosahedral, membrane-containing dsDNA viruses exemplified by bacteriophage PRD1 (Bamford et al., 2002, 2005a; Benson et al., 2004). The first member of this lineage from the archaeal domain is the *Sulfolobus* turreted icosahedral virus (STIV) that infects the crenarchaeal host *Sulfolobus solfataricus* (Khayat et al., 2005; Rice et al., 2004).

Here we report the overall distribution of the proteins in the SH1 virion. To determine the localization of the different proteins and the membrane, two different quantitative dissociation conditions were identified. The virion was dissociated into soluble capsid-associated proteins and a lipid core containing the membrane proteins and the dsDNA genome.

Results

SH1 has an internal lipid membrane

To obtain enough material for the dissociation studies, we further optimized the production and purification of the virus. Modifications of the different steps of the purification protocol improved the yield considerably leading to specific infectivity of $4–6\times10^{11}$ PFU/A_{260} or $4.5–6.5\times10^{12}$ PFU/mg of protein in the 2× purified virus preparation. To dissociate the SH1 virion in a controlled manner, different conditions were tested, and the outcome was analyzed by rate zonal centrifugation followed by determination of the protein content in each fraction by tricine-SDS-PAGE. The conditions used were heating, freezing and thawing, low ionic strength and different concentrations of DTT, mercaptoethanol, urea and SDS as well as treatment with proteases.

Controlled dissociation of the virus was obtained only with treatment with 3 M urea or using low ionic strength (Figs. 1A and 2A). In low ionic strength conditions, structural proteins VP2, VP3, VP4, VP6, VP7 and VP9 were quantitatively released as soluble proteins from the virion. In addition, DNA-less particles sedimenting slower than virions were detected (Fig. 1A). In these dissociation conditions approximately, 40–50% of VP5 protein stayed associated with the particle with the rest being released. Some 15% of VP1 was also retained with the particle. Lipid analysis (Fig. 1B) showed that the large majority of viral lipids were retained with the sedimenting particle in similar proportions as in the native SH1 virion. The density of the sedimenting particle in sucrose was 1.15 g/ml, and vesicular material was observed in thin section electron microscopy (Fig. 1C). The density of the intact virion is 1.28 g/ml (Porter et al., 2005).

Treatment with urea gave similar dissociation products as the low ionic strength conditions except for protein VP1 and the genomic DNA. Following urea treatment VP1 and DNA stayed almost entirely with the slowly sedimenting particle (Fig. 2A). Moreover, the lipid content of the particle obtained by urea dissociation was confirmed to be comparable to the lipids in the virion and in the membrane vesicles obtained by reducing the salt concentration (Fig. 2B). We designate the proteinaceous DNA- and lipid-containing particle as the lipid core (LC) in analogy to the corresponding particle obtained from phage PM2 (Kivelä et al., 2002).

SDS-PAGE analysis of LC showed that VP10 and VP12 were the major proteins associated with it. In addition, three minor protein bands around 37 kDa were also detected (Figs. 1A and 2A). Their identities were determined by N-terminal sequencing (low ionic strength conditions) utilizing the sequence information (Bamford et al., 2005b). The fastest migrating band was VP5 (TISAP). The protein band above VP5 gave a sequence of ADQTQEYTI identifying it as VP4. N-terminal sequencing of the uppermost band gave a peptide sequence of GVKDQIRDL-DEYQAQ, which corresponds to the deduced N-terminal sequence of VP13. These identities were further confirmed by mass spectrometry finger printing (data not shown).

The distribution of the SH1 proteins between the capsid and the lipid core (dissociated in low ionic strength conditions) were quantified as shown in Fig. 3. Assuming equal binding of Coomassie blue and taking into account the molecular masses (Table 1), it appeared that the three most abundant proteins in the capsid fraction VP3, VP4 and VP7 were present at approximate ratios of 0.24:0.6:1, respectively. The amount of VP13 migrating at the same position as VP4 has been taken into

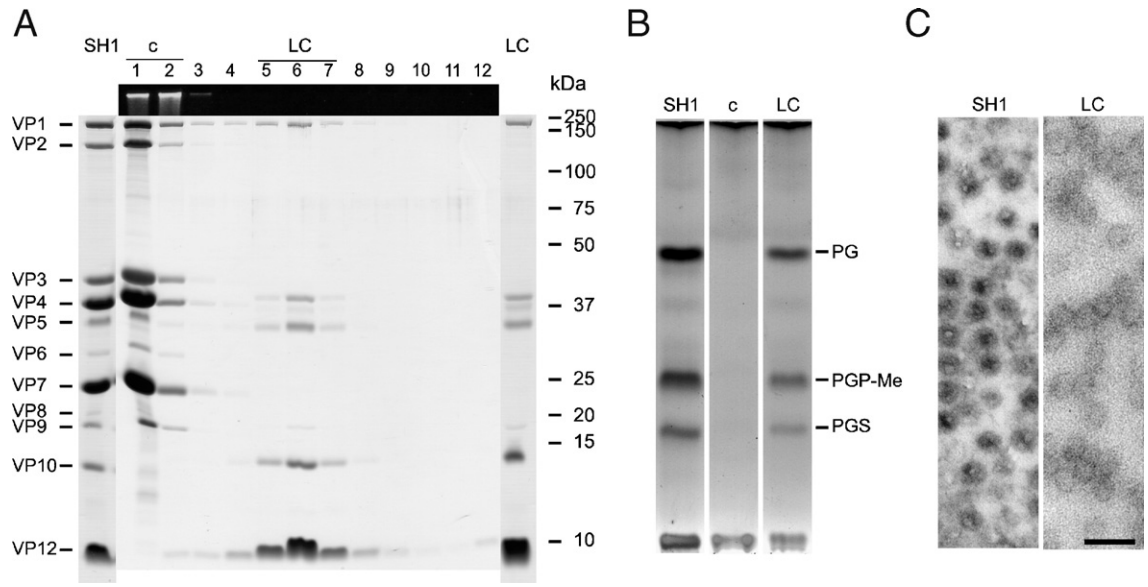

Fig. 1. Dissociation of SH1 virion in low ionic strength and separation of the products by rate zonal centrifugation. (A) SH1 proteins analyzed in Coomassie blue-stained tricine-SDS-PAGE. The sedimentation is from left to right. The stacking gel stained with ethidium bromide indicates the presence of DNA. The soluble fraction (c) and a light scattering zone (LC) are indicated. Concentrated preparation of the LC fraction is shown as the rightmost lane. SH1 virion proteins are indicated on the left and the molecular masses of the standard proteins on the right. (B) Lipid analysis of the virion (SH1), the soluble protein fraction (c) and a particle (LC) performed by thin-layer chromatography. The fractions used for lipid analysis are indicated by bars in panel A. PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerophosphate methyl ester; PGS, phosphatidylglycerosulphate. (C) Thin section electron microscopy of SH1 virion (SH1) and the particle obtained from the light scattering zone (LC). Scale bar represents 100 nm.

account in the calculations. The largest SH1 proteins VP1 and VP2 with monomeric molecular masses of 152 kDa and 81 kDa, respectively are present in minor amounts having a ratio of about 0.04:0.06:1 (VP1:VP2:VP7). VP12 is the most abundant protein in LC contributing almost 70% of the total proteins in it.

Analysis of SH1 capsid-associated proteins by sedimentation and gel filtration

The non LC-associated SH1 proteins released by low ionic strength buffer (Fig. 1A, fraction 1) were subjected to rate zonal centrifugation analysis in a 5–20% sucrose gradient. In Fig. 4 the sedimentation time (48 h) was set to display all the proteins. Sedimentation time of 66 h was used to maximize the separation of protein VP4 and VP7 with the consequence that VP1 is in the pellet (data not shown). Fractions of the rate zonal gradient were further analyzed by gel filtration, and the obtained chromatography fractions were subjected to SDS-PAGE analysis. Two such gel filtration analyses of the fractions are shown in Figs. 5A and B as examples illustrating the separation of proteins VP2, VP3, VP4, VP6, VP7 and VP9.

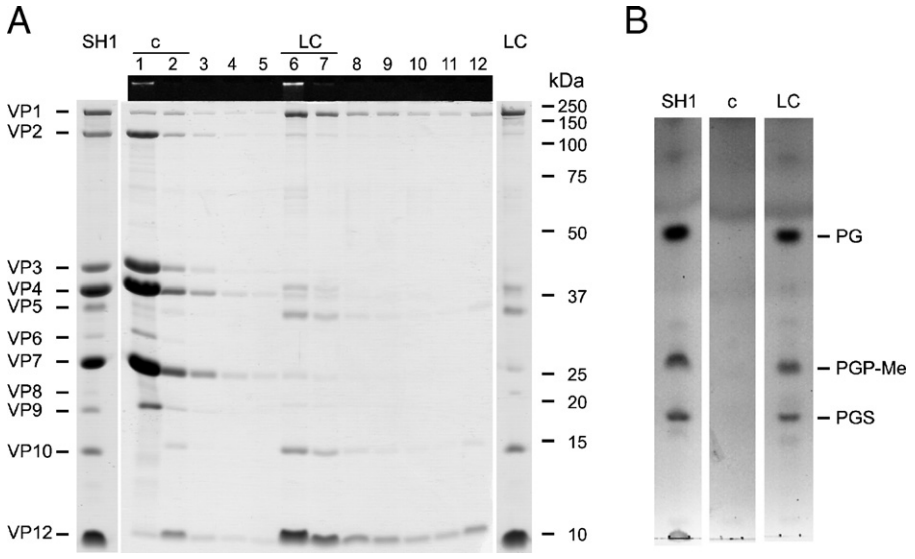

Fig. 2. Dissociation of SH1 virion in 3 M urea. The data are presented as in Fig. 1.

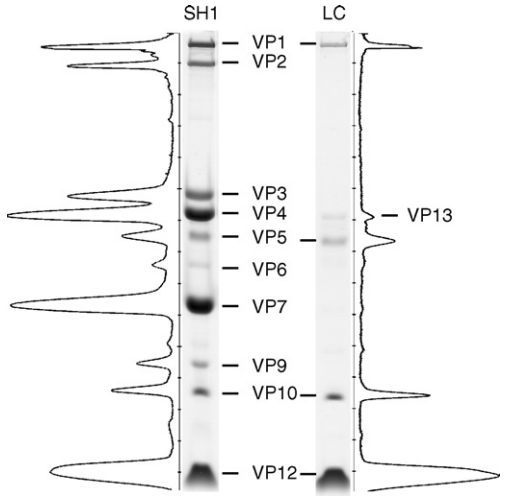

Fig. 3. Quantification of the SH1 and lipid core (LC) proteins. The scanned and analyzed Coomassie blue stained polyacrylamide gels are shown next to the profiles. The most abundant SH1 proteins are indicated.

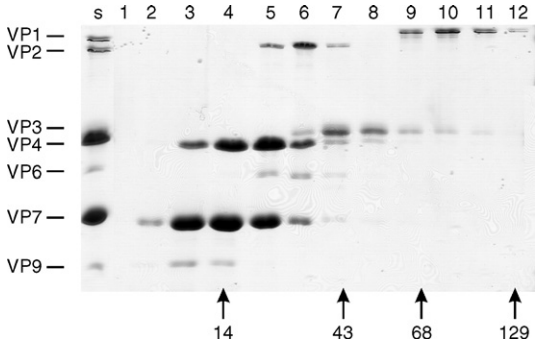

Fig. 4. Sedimentation (48 h) of the soluble proteins obtained with treatment in low ionic strength (Fig. 1) was analyzed by rate zonal centrifugation (5%–20% sucrose) as described in Materials and methods. The sedimentation fractions were analyzed in Coomassie blue-stained polyacrylamide gels. Relevant SH1 proteins are indicated on the left. S = concentrated starting material. Sedimentation standards (in kDa) are shown below.

Sedimentation analysis showed that all the major capsid proteins, i.e., VP1, VP2, VP3, VP4, VP7 and minor proteins VP6 and VP9 were found in specific positions (Fig. 4). However, proteins VP4 and VP7 also overlapped indicating that there might be both homo- and heteromultimers in addition to monomeric forms. The results for molecular mass determinations using both sedimentation and gel filtration in low salt conditions are presented in Table 1. There is a significant difference in the mass determination for proteins VP1, VP2, VP4 and VP7 between the two methods. Only VP3 gave consistent results throughout the experiments. The gel filtration analysis was also carried out in 500 mM NaCl with identical results (data not shown) showing that the proteins were insensitive to salt concentration in the conditions used. These results reflect considerable differences in the hydrodynamic radii of the proteins and consequently elongated shapes.

Discussion

Halovirus SH1 may have a similar overall structure to Sulfolobus turreted icosahedral virus (STIV) and bacteriophages PRD1, PM2, Bam35 as well as *Paramecium bursaria* chlorella virus 1 (PBCV-1) namely a dsDNA genome encapsidated into a membrane vesicle residing inside an icosahedral protein coat (Abrescia et al., 2004; Bamford et al., 2005b; Cockburn et al., 2004; Huiskonen et al., 2004; Khayat et al., 2005; Laurinmäki et al., 2005; Nandhagopal et al., 2002; Porter et al., 2005). It has also been proposed that both PRD1 and STIV belong to a lineage of viruses with common architectural principles and having members infecting hosts in the three different domains of life and may have a common ancestry (Bamford et al., 2005a; Benson et al., 2004). The three dimensional structure of the STIV coat protein has recently been resolved to 2.0 Å resolution (Khayat et al., 2005) showing that it has the typical double-barrel fold found in the PRD1-adenovirus lineage of viruses (Benson et al., 2004). Currently it is not known whether PM2 shares more detailed structural similarities to these viruses.

To verify that SH1 has such an overall structural arrangement, we performed controlled dissociation experiments. Most of our trials led to unspecific disruption, but both low ionic strength and urea treatments revealed a lipid vesicle and released soluble capsid-associated proteins. When using urea the viral genome resided within the emerged membrane vesicle, whereas the reduction of salt also released the genome, leaving an empty vesicle behind (Figs. 1 and 2). These results confirm that there is an internal membrane with a specific protein composition within the SH1 virion, analogous to PRD1, Bam35, PM2, PBCV-1 and STIV.

The major protein associated with the membrane, VP12, has two predicted transmembrane helices (Bamford et al., 2005b), strongly suggesting that it is an integral membrane protein. The other proteins detectably associated only with the membrane fraction were VP10 and VP13. According to the sequence data, VP13 is an 8.8-kDa protein. It has been identified before on the basis of mass spectrometric analysis from the position appropriate to its predicted size. Thus, it was unexpected to find it at a position corresponding to approximately 37 kDa in size. Protein VP5 was almost equally distributed between the membrane and the soluble protein fraction, and there were residual amounts of VP4 in the membrane fraction, indicating their proximity to the membrane.

Table 1
Molecular masses of SH1 capsid associated proteins determined by sedimentation as well as gel filtration and compared to the calculated monomer mass

Protein	Molecular mass (kDa)		
	Monomer	Sedimentation	Gel filtration
VP1	152.3	~85	~550–600
VP2	81.1	~25	~550–600
VP3	37.5	~35	~40
VP4	25.7	~15	~200/100
VP6	25.0	~25	~60
VP7	20.0	~15	~170/80
VP9	16.5	<14	~50

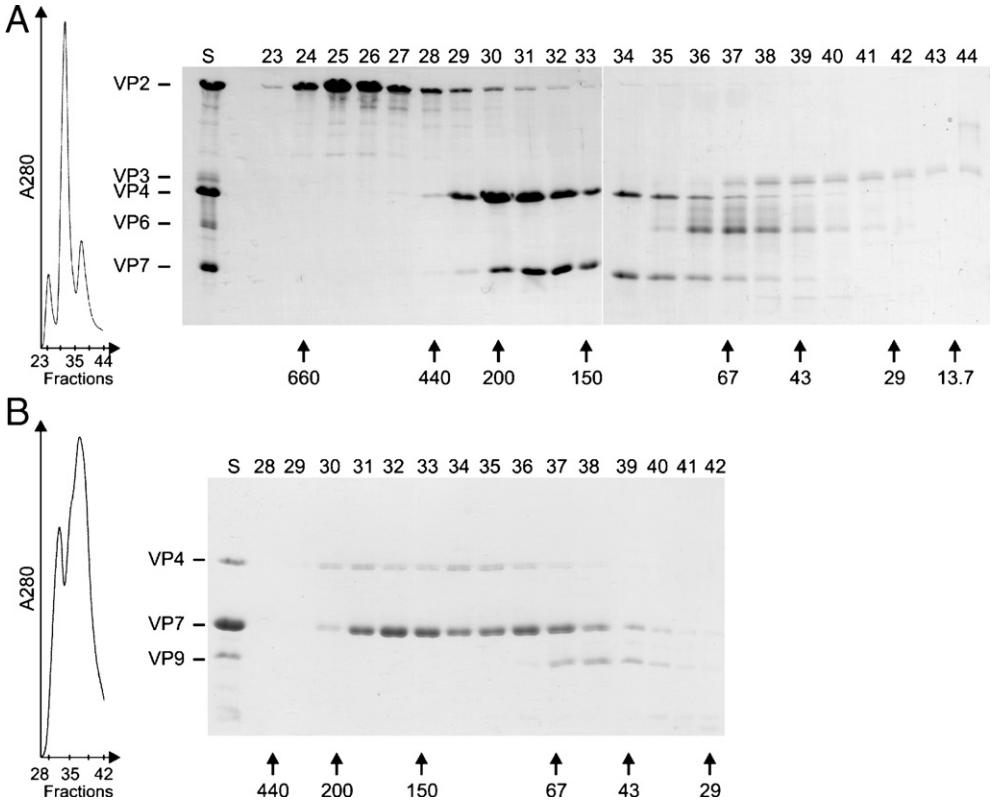

Fig. 5. Examples of gel filtration analysis of fractions obtained from the rate zonal centrifugation (Fig. 4). (A) Elution profile and SDS-PAGE analysis of fraction 6 from the 48-h centrifugation (Fig. 4) and fraction number 8 from the 66-h centrifugation (data not shown). (B) Elution profile and SDS-PAGE analysis of fractions 3–4 from the 66 h centrifugation (data not shown). S=starting material. Gel filtration standards (in kDa) are shown below.

The number of capsid-associated SH1 proteins is high (at least proteins VP1, VP2, VP3, VP4, VP5, VP6, VP7, VP9) as indicated by the dissociation experiments. Consequently, the capsid structure appears to be complex. The largely deviating results obtained by sedimentation and gel filtration analyses (Table 1) indicate that minor proteins VP1 and VP2 are highly asymmetric. Such proteins are often located in spike structures (Caldentey et al., 2000). As SH1 seems to have spikes at the fivefold vertices (H. Jäälinoja and S. Butcher, personal communication) we propose a location of VP1 and VP2 at the vertices forming spikes. Most of the VP1 stays associated with the LC when urea is used for dissociation. This suggests that although VP1 is not predicted to contain any transmembrane helices it is associated with the membrane as a peripheral membrane protein. Dissociation of the virions in low ionic strength released the majority of VP1 from the LC. At the same time the genomic DNA was released. It is tempting to speculate that this 152-kDa VP1 serves a cap-like function, but the two events may also be independent. VP1 has been previously shown to form cross-linked homomultimeric complexes (most probably trimers, Bamford et al., 2005b). The amino acid sequence of VP2 shows typical patterns of fiber-like proteins (Bamford et al., 2005b) in line with results obtained here.

It was previously observed, by analyzing naturally occurring cross-linked virion proteins, that VP4 and VP7 form homo- and heteromultimers in addition to their monomeric forms (Bamford et al., 2005b). The sedimentation analysis performed here was able to separate these proteins although their broad sedimenting zones also overlapped (Fig. 4). Gel filtration analyses of the sedimentation gradient fractions showed that there are two species of both VP4 and VP7 (Fig. 5, Table 1) one possibly representing an elongated monomer and a multimer. We were not able to detect separate VP4-VP7 heteromultimers previously seen in cross-linked complexes (Bamford et al., 2005b). The abundance and tight associations of these two proteins predict them to form the icosahedral shell. The quantification of the SDS-PAGE band intensities (Fig. 3) suggests that there are two VP7 proteins for each VP4. This conclusion, however, is obtained by assuming equal stain binding to both proteins. The third most abundant shell-associated protein VP3 clearly behaved as a globular monomer. Whether this protein is part of the coat structure remains to be seen. The analyses were conducted in low salt (150 mM) alien to halophilic proteins. Since no aggregation was detected, denaturation due to low salt seems unlikely. There are also minor structural proteins, VP6, VP8, VP9, VP11, VP14 and possibly VP15 (Bamford et al., 2005b) that were not included in this discussion due to their insignificant amount (although maybe not insignificant function).

In conclusion, the dissociation experiments and analyses of the dissociation products confirm the intricate architecture of the SH1 capsid. The overall architecture of the virion (an innermost proteinaceous lipid vesicle enclosing the genome, covered by a protein shell with possible spike structures)

resembles those of PRD1, Bam35, PBCV-1, PM2, and STIV. If SH1 belongs to the suggested PRD1-adenovirus lineage, the capsid architecture is more elaborate than in other previously well-studied viruses belonging to this lineage. SH1 seems to have at least two proteins serving the same function as one coat protein in the other viruses. Interestingly, the sum of VP4 and VP7 masses corresponds to the masses of PRD1 and STIV coat proteins containing two beta barrels. It will be intriguing to learn how closely SH1 resembles PRD1 and the other members of this lineage in the detailed structure.

Materials and methods

Cells, viruses, and purification of viruses

H. hispanica (ATCC 33960) was originally isolated from a solar saltern in Alicante, Spain (Juez et al., 1986). SH1 was isolated from a salt pond on Rottnest Island on the western coast of Australia using *H. hispanica* as a host (Porter et al., 2005). *H. hispanica* and SH1 were cultured in modified growth medium (MGM) containing 18%, 20%, or 23% (w/v) artificial salt water (SW) (Nuttall and Dyall-Smith, 1993; The Halohandbook,http://www.microbiol.unimelb.edu.au/people/dyallsmith/HaloHandbook/). All media were prepared in tap water.

Viruses were purified either from plate lysates or from liquid cultures as described by Porter et al. (2005) with modifications. The buffer used throughout the virus purification was changed to 18% (w/v) SW. Aerated mid-exponential growth phase culture of *H. hispanica* (cell density of approximately 5×10^8 CFU/ml) was infected using a MOI of 40 and grown at 37 °C until cell lysis (~5.5 h) occurred. The lysate was treated with DNase I for 30 min at 37 °C (70 μg/ml; Sigma). After removal of the cell debris (Sorvall SLA3000, 8000 rpm, 20 min, 4 °C), viruses were concentrated with 10% (w/v) polyethylene glycol 6000, collected (Sorvall SLA1500, 10,500 rpm, 40 min, 4 °C), and resuspended on ice in 18% (w/v) SW (to 13 ml/liter of lysate). Aggregates were removed (Sorvall SS34, 7000 rpm, 10 min, 4 °C), and viruses were purified by rate zonal centrifugation in a linear 5% to 20% (w/v) sucrose gradient in 18% (w/v) SW (Sorvall AH629, 22,000 rpm, 1:30 h, 20 °C) to obtain 1× purified virus particles. These were further purified by isopycnic centrifugation in a CsCl gradient in 18% (w/v) SW with an average density of 1.3 g/ml (Sorvall AH629, 20,000 rpm, 16 h, 20 °C). The equilibrated virus band (2× purified) was diluted in 18% SW devoid of NaCl (volume ratio 1:2) prior to concentration by differential centrifugation (Sorvall T647.5, 32,000 rpm, 3 h, 20 °C). The viruses were resuspended on ice in SH1 buffer (40 mM Tris–HCl, pH 7.2, 1 M NaCl, 40 mM $MgCl_2$).

Dissociation of viral particles

The different conditions used for virus dissociation are as follow:

(1) Virus particles (~1 mg of protein/ml) in SH1 buffer were treated with 3 M urea for 15 min at 22 °C.
(2) Dissociation in low ionic strength conditions was done by either (a) resuspending purified virus particles (~1 mg of protein/ml) in 10 mM Tris–HCl, pH 7.2, 1 mM EDTA for 1 h at 22 °C or (b) diluting purified SH1 virus particles (1:13, ~1 mg of protein/ml) in 20 mM Tris–HCl, pH 7.2, 150 mM NaCl and incubating for 1 h at 22 °C.
(3) Other conditions used for dissociation experiments of purified SH1 (1 mg/ml) involved 0.5–2% sodium dodecylsulphate (SDS), 0.5–80 mM dithiothreitol (DTT) and 0.5–5% mercaptoethanol in SH1-buffer combined with temperatures of either 4 °C or 37 °C.
(4) For protease treatments SH1 (1 mg of protein/ml) was incubated with trypsin (0.1 mg/ml, Boehringer Mannheim), proteinase K (0.1 mg/ml, Roche), pronase (1 mg/ml, Boehringer Mannheim) or bromelain (7 mg/ml, ICN Biomedicals) in SH1-buffer containing 2 mM $CaCl_2$. All samples were incubated at 37 °C for 2 h.

After all dissociation experiments the results were analyzed by rate zonal sucrose gradient centrifugation of either 5% to 20% (w/v, Sorvall TH641, 22,000 rpm, 1 h 10 min–1 h 50 min, 20 °C) or 10% to 30% (w/v, Sorvall TH641, 30,000 rpm, 2 h, 20 °C) in SH1 buffer or in low ionic strength buffers.

Analysis of dissociation products

Soluble proteins obtained by dissociation of SH1 with 20 mM Tris–HCl, pH 7.2, 150 mM NaCl were separated from the membrane moiety of the virus by rate zonal centrifugation (see above). The soluble proteins on the top of the gradient were collected, concentrated with Amicon Ultra 15 concentrators (MWCO 5000) and subjected to rate zonal centrifugation in a linear 5% to 20% sucrose gradient in 20 mM Tris–HCl pH 7.2, 150 mM NaCl (Sorvall TH641, 36,000 rpm, 48 h or 66 h, 15 °C). Lysozyme (14.3 kDa), ovalbumin (43 kDa), bovine serum albumin (BSA, 68 kDa), and PRD1 P3 trimer (129 kDa) were used as markers. The sedimentation gradient fractions were pooled, concentrated with Amicon Ultra 4 concentrators (MWCO 10,000) and analyzed by gel filtration (Tricorn Superdex 200 10/300 GL, GE Healthcare). The column was equilibrated with 20 mM Tris–HCl pH 7.2 containing either 150 mM or 500 mM NaCl. Blue dextran (2000 kDa), thyroglobulin (660 kDa), apoferritin (440 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA, ovalbumin, carbonic anhydrase (29 kDa), and RNase A (13.7 kDa) were used as molecular mass markers. Fractions containing protein were subjected to SDS-PAGE analysis.

For lipid analysis, gradient fractions containing SH1 dissociation products (low ionic strength and urea treatments) were extracted by a standard procedure (Folch et al., 1957) modified for halophiles (Kates, 1972), dissolved in chloroform/methanol (9:1, v/v) and stored at −20 °C. The lipid extracts were qualitatively analyzed by thin-layer chromatography (silica gel 60 plates, Merck) using chloroform/methanol/90% acetic acid (65:4:35, v/v) as solvent (Corcelli et al., 2000). Lipids extracted from SH1 and *H. hispanica* were used as standards. The lipids were visualized with iodine vapor.

Relative abundances of lipids in the gradient fractions were determined by electro-spray ionization mass spectrometry as previously described (Bamford et al., 2005b).

The density of the slowly sedimenting particle after dissociation in low ionic strength (20 mM Tris–HCl pH 7.2, 150 mM NaCl) was determined in a linear 20% to 70% (in the buffer used for dissociation) sucrose gradient (Sorvall TH641, 22,000 rpm, 18 h, 20 °C).

Protein analyses

Protein concentrations were determined by the Bradford (1976) method using bovine serum albumin as a standard. Proteins were analyzed by 15% SDS-polyacrylamide gel electrophoresis (PAGE) or by 14% tricine-SDS-PAGE (Schägger and von Jagow, 1987). Determination of N-terminal amino acid sequences was conducted with proteins separated by tricine-SDS-PAGE as described earlier (Bamford et al., 2005b). Mass spectrometry was used to identify proteins separated by tricine-SDS-PAGE. For analysis by mass spectrometry the proteins were in-gel digested with trypsin and analyzed by matrix-assisted laser desorption–ionization time of flight (MALDI-TOF) MS for mass fingerprinting as previously described (Bamford et al., 2005b). Identification of proteins by N-terminal sequencing as well as analysis with mass spectrometry was conducted in the Protein Chemistry Research Group and Core Facility, Institute of Biotechnology, University of Helsinki.

Quantification of the capsid- and LC-associated proteins was conducted from Coomassie stained, scanned gels with TINA 2.09c software (Raytest, Isotopenmessgeräte GmbH, Staubenhardt, Germany). The relative ratios of the most abundant capsid proteins were obtained by setting the molecular mass calibrated intensity of the most abundant protein VP7 as 1. The ratios obtained for proteins VP1, VP2, VP3 and VP4 were calculated by dividing the intensities calibrated by the molecular mass of the protein by the calibrated intensity of VP7.

Electron microscopy

SH1 virus preparation (1× purified) was treated in low ionic strength buffer as described above except that 20 mM Tris–HCl pH 7.2 was replaced with 20 mM MES pH 6.7 throughout the experiments. After the sucrose gradient centrifugation the light scattering zone was collected and concentrated (Sorvall TH641, 30,000 rpm, 1 h, 20 °C). The control SH1 virions were incubated in 20 mM MES pH 6.7, 1 M NaCl, 40 mM $MgCl_2$ buffer at 4 °C for 2 h and concentrated as described above. Both samples were fixed with 3% glutaraldehyde (v/v) for 30 min at 22 °C. Thin-section electron microscopy was performed as previously described (Bamford and Mindich, 1980) in the Electron Microscopy Unit, Institute of Biotechnology, University of Helsinki.

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