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Macromolecular mass spectrometry and electron microscopy as complementary tools for investigation of the heterogeneity of bacteriophage portal assemblies

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Abstract

The success of electron-cryo microscopy (cryo-EM) and image reconstruction of cyclic oligomers, such as the viral and bacteriophage portals, depends on the accurate knowledge of their order of symmetry. A number of statistical methods of image analysis address this problem, but often do not provide unambiguous results. Direct measurement of the oligomeric state of multisubunit protein assemblies is difficult when the number of subunits is large and one subunit renders only a small increment to the full size of the oligomer. Moreover, when mixtures of different stochiometries are present techniques such as analytical centrifugation or size-exclusion chromatography are also less helpful. Here, we use electrospray ionization mass spectrometry to directly determine the oligomeric states of the *in vitro* assembled portal oligomers of the phages P22, Phi-29 and SPP1, which range in mass from 430 kDa to about 1 million Da. Our data unambiguously reveal that the oligomeric states of Phi-29 and SPP1 portals were 12 and 13, respectively, in good agreement with crystallographic and electron microscopy data. However, *in vitro* assembled P22 portals were a mixture of 11- and 12-mer species in an approximate ratio of 2:1, respectively. A subsequent reference-free alignment of electron microscopy images of the P22 portal confirmed this mixture of oligomeric states. We conclude that macromolecular mass spectrometry is a valuable tool in structural biology that can aide in the determination of oligomeric states and symmetry of assemblies, providing a good starting point for improved image analysis of cryo-EM data.

Keywords: Phage; Portal; Assembly; Stochiometry; Electrospray mass spectrometry; Non-covalent complexes

1. Introduction

The development of electrospray ionization coupled to mass spectrometry has enabled the analysis of very large intact protein complexes even when they are held together by weak non-covalent interactions. Together with equally spectacular advances in mass spectrometric instrumentation a new field has emerged, sometimes termed native protein mass spectrometry, that focuses on the structural and functional analysis of the dynamics and interactions occurring in protein complexes. In the past years, several important technological innovations have been reported that have enabled exciting applications ranging from the detailed study of equilibria between different quaternary structures as influenced by binding of substrates or cofactors to the

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analysis of intact nano-machineries, such as whole virus particles, proteasomes and ribosomes (Benjamin et al., 1998; Videler et al., 2005; Bothner and Siuzdak, 2004; Loo et al., 2005). For the analysis of intact protein complexes by mass spectrometry the sample of interest is electrosprayed from an aqueous solution of a volatile buffer such as ammonium acetate. Desolvation of the protein assemblies in the ion source interface generates multiply charged ions of the intact complexes for analysis by the mass spectrometer. Mass spectrometric detection of the assemblies has made it possible to obtain accurate information about protein complex stoichiometry, stability and dynamics (Heck and Van den Heuvel, 2004; Loo, 1997; van den Heuvel and Heck, 2004; Ilag et al., 2005), through which, for instance, the folding cycle of the GroEL-gp31 machinery could be monitored while folding the bacteriophage gp23 capsid protein (van Duijn et al., 2005). The detectable mass range in electrospray ionization mass spectrometry exceeds several million Da, allowing the analysis of species as big as ribosomes and viruses (Benjamin et al., 1998; Bothner and Siuzdak, 2004). Therefore, macromolecular mass spectrometry lends itself as an excellent tool to study protein complex assembly and, in particular, virus assembly, where major questions focus on the early multi-protein intermediates of assembly, or the stoichiometries of subcomplexes, such as the portal. We have used macromolecular mass spectrometry to examine the stoichiometry of several bacteriophage portal complexes, which are the molecular motors that drive the DNA packaging.

dsDNA viruses of orders *Caudovirales* and *Herpesviridae* rely on an active packaging mechanism for encapsidation of their genomes (Hohn, 1976). Genomic DNA is packaged into a pre-formed icosahedral protein shell (termed a procapsid) against a large internal force in an ATP-dependent manner to near crystalline density. The molecular motors that perform this task constitute a distinct structural class (Moore and Prevelige, 2002b). The packaging motor of Phi-29 is the most powerful molecular motor known to-date and is capable of creating up to 57 pN of force (Smith et al., 2001).

The DNA packaging motors consist of two principal parts: the terminase complex and the portal complex or head-to-tail connector (Fujisawa and Morita, 1997). Terminase is an ATPase that cuts the concatameric DNA at a particular recognition sequence, targets it to portal and, possibly, constitutes an integral part of the packaging complex translating the energy of ATP hydrolysis into the inward movement of the DNA (Catalano, 2000; Guo et al., 1987; Mitchell and Rao, 2006; Grimes et al., 2002; Morita et al., 1993). The portal complex is a ring-like structure located at one icosahedral vertex of the capsid. It performs multiple tasks during the viral lifecycle: (a) it comprises the conduit through which DNA passes during packaging and infection (Simpson et al., 2000); (b) it acts as a sensor in controlling the amount of DNA packaged (Casjens et al., 1992; Lander et al., 2006; Tavares et al., 1992); (c) together with other structural proteins it prevents packaged DNA from leaking out of the capsid prior to infection (Strauss and King, 1984; Orlova et al., 2003); (d) it possibly plays an active role in DNA packaging and (e) it mediates the connection between the phage head and tail (Ackermann, 1998).

The portal occupies one of the 12 pentameric vertices of icosahedral viral capsid. All portals studied *in situ* or in complex with other viral structural proteins are dodecameric (12-mer) ring-like structures composed of the portal protein (Lurz et al., 2001; Carrascosa et al., 1985; Donate et al., 1988; Kochan et al., 1984; Jiang et al., 2006; Tang et al., 2005; Chang et al., 2006; Agirrezabala et al., 2005a), which share significant overall structural similarity despite the lack of any noticeable sequence similarity (Orlova et al., 2003; Badasso et al., 2000; Guasch et al., 2002; Simpson et al., 2000; Agirrezabala et al., 2005b; Trus et al., 2004). Structurally, portals can be divided into three domains: the stalk, the wings and the crown (Fig. 1), with the greatest morphological similarity between portals found in the stalk region. The narrowest part of the central channel has a diameter of 30 Å, slightly larger than the diameter of DNA.

The tail attaches to the narrow end of the portal (stalk region) after DNA packaging has been terminated. While interaction of the 12-fold portal with the 6-fold tail generates symmetry ambiguity, since the 6-fold can attach in one of two registers, the replacement of a pentameric icosahedral vertex by a dodecameric portal complex represents a more obvious symmetry mismatch, and implies that there can be no regular interactions between the capsid and the portal (Jiang et al., 2006; Lander et al., 2006). This observation led to the proposal that the portal sits in the capsid like a bearing and that it might function like one, i.e. the whole packaging assembly might work as a rotary motor (Hendrix, 1978). Supporting that model, the high resolution structure of phage Phi-29 portal has revealed that the surface opposing the capsid is hydrophobic and may form a greasy interface that would facilitate rotation (Guasch et al., 2002; Simpson et al., 2000).

The lack of regular portal/capsid interactions in the final structure raises the intriguing question of how portal and capsid subunits co-assemble into the well-defined capsid structure. Assembly occurs in a two-step process. In the first step the capsid protein co-polymerizes with a scaffolding protein to form a procapsid. In the second step, genomic DNA is packaged into the procapsid triggering a structural transformation known as maturation. The portal protein is not required for procapsid assembly, nor can the portal be incorporated into already assembled procapsids (Moore and Prevelige, 2002a; Newcomb et al., 2005), suggesting that important interactions between portal and capsid proteins are only formed during procapsid assembly.

Portal protein subunits readily assemble both *in vivo* and *in vitro*. Surprisingly, however, *in vitro* the portal protein subunits do not necessarily assemble into the same oligomeric state as they would *in situ*, i.e., as part of the complete bacteriophage structure. Whereas exclusively dodecameric

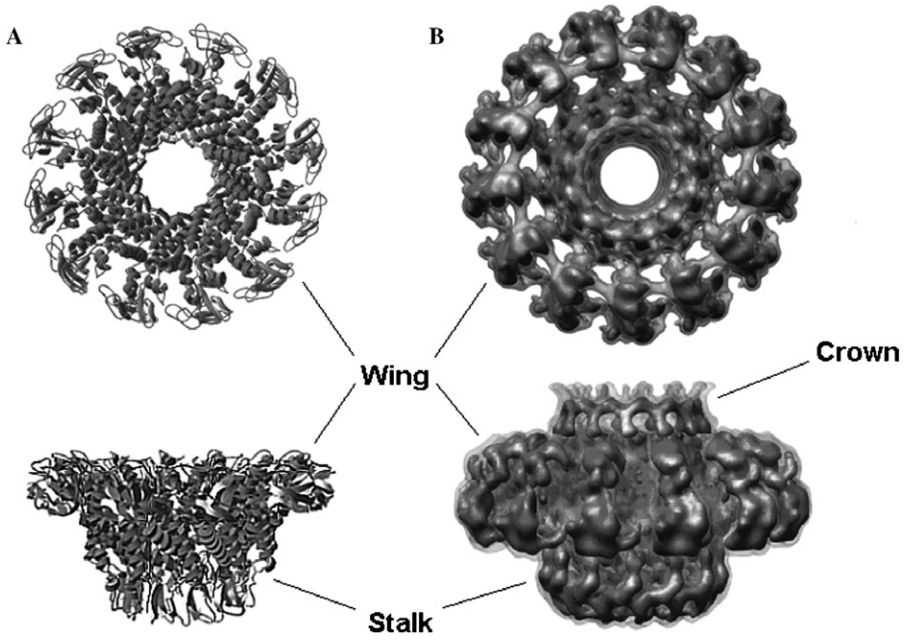

Fig. 1. Models of the structures of phage portals (not to scale). (A) Crystal structure of the reconstituted Phi-29 portal (Simpson et al., 2000), top view (top), side view (bottom). This crystal structure indicates clearly that the Phi-29 portal forms a dodecameric ring structure with an α-helical, central spring-like stalk domain. DNA passes through the central channel during packaging and upon infection. (B) Cryo-electron microscopy image reconstruction of the 13-mer of the reconstituted SPP1 portal (EBI Accession Code EMD-1020, Orlova et al., 2003), top view (top), side view (bottom). These images clearly reveal that *in vitro* the SPP1 portal assembles into 13-mers. Its overall shape (and of other studied portals) is very similar to that of the Phi-29 portal. SPP1 portal image was created using UCSF Chimera software.

portal complexes are formed *in situ*, it has been shown that *in vitro* other stochiometries can also occur (T7 (Agirrezabala et al., 2005b; Cerritelli and Studier, 1996; Kocsis et al., 1995), SPP1 (Jekow et al., 1998; Orlova et al., 1999; van Heel et al., 1996), T4 (Rishovd et al., 1998), T3 (Valpuesta et al., 2000), Herpes (Trus et al., 2004)). This is rather surprising since this suggests that the dodecameric arrangement of portal protein seen universally *in situ* is not necessarily strongly favored thermodynamically or kinetically and implies that correct portal assembly is either coupled to procapsid assembly, or requires additional factors, i.e., proteins, not present in the *in vitro* reconstitution. Therefore, it is very interesting to unravel the mechanism of portal assembly.

To date only one high-resolution crystal structure of a phage portal has been published—that of Phi-29 (Guasch et al., 2002; Simpson et al., 2000) with the bulk of the structural information about this important group of molecules being obtained by electron microscopy and image analysis. To obtain the best resolution reconstructions it is advantageous to know the oligomeric state of the species present; the best results are obtained when a homogenous population of assemblies is present. Using the electron microscope to test for a homogenous population, however, is challenging. While the stoichiometry of smaller assemblies may be analyzed by size-exclusion chromatography or analytical ultracentrifugation measurements, intermediate molecular weight complexes with high order oligomerization (for example, 11, 12, and 13 subunits), are probably among the most difficult to analyze (Kocsis et al., 1995). A direct method of determining the oligomer composition of such species would be a significant aide to structural studies on macromolecular complexes.

In this work, we have applied electrospray ionization mass spectrometry to determine the oligomeric states of *in vitro* reconstituted portals, of the phages Phi-29, P22, and SPP1. Our mass spectrometric data unambiguously determine the oligomeric states of Phi-29 and SPP1 portals as being 12 and 13, respectively. P22 portals, however, are a mixture of 11- and 12-mer species in an approximate ratio of 3:1, respectively. A subsequent model independent rotational averaging analysis of electron microscopy images of P22 portal confirmed semi-quantitatively this mixture of oligomeric states. We conclude therefore that macromolecular mass spectrometry is a very valuable tool in structural biology as it can determine the oligomeric states and symmetry of assemblies, which can be used to facilitate image analysis in cryo-electron microscopy.

2. Results

2.1. Preparation of portal assemblies

All portal proteins studied were over-expressed in *Escherichia coli* and purified by previously published procedures as described in Section 4. The proteins were greater than 95% pure as judged by SDS–PAGE. Following *in vitro* reconstitution the approximate molecular weight of each of the portal assemblies was first estimated by gel filtration chromatography under buffer conditions known to

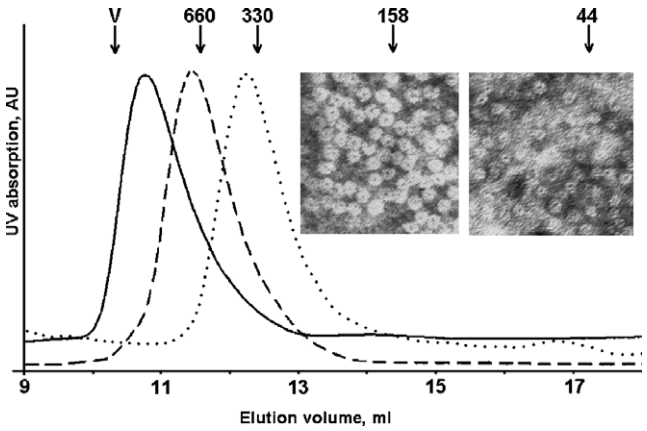

Fig. 2. Size exclusion chromatography of Phi-29, SPP1 and P22 portal assemblies in Tris buffer solution. The solid line is of P22fl-his; the dashed line represents SPP1, and the dotted line Phi-29. The position of elution of marker proteins is, together with their molecular weight, indicated above the chromatogram (V—void volume). From these chromatograms it may be concluded that the Phi-29 portal leaves the column at ~400 kDa; SPP1 and P222 elute between the 660 kDa and included volume markers . The insets show electron microscopy images of uranyl acetate stained portals, clearly revealing ring-like structures for on the left P22fl-his portal and on the right SPP1.

stabilize the higher order assemblies (Fig. 2). All portal complexes eluted as single peaks in the high molecular weight range, suggesting their successful assembly. The Phi-29 portal complex eluted at an elution volume corresponding to a molecular weight of approximately 400 kDa. Both SPP1 and P22 portals eluted between the column's void volume and elution volume of 660 kDa marker protein suggesting that both portals proteins are in high oligomeric states. Additionally, the presence of ring-like structures in the reconstituted preparations of SPP1 and P22 portal assemblies could be confirmed by negative stain electron microscopy (Fig. 2, insets).

In our preparation, the full length P22 portal protein, designated P22fl-his, has an eight amino acid His-tag extension at the C-terminus. It has been previously shown that phage particles with this specific portal can replicate and have a morphology indistinguishable from the wild type phage (Moore and Prevelige, 2001). However, the burst size of the mutant phage is somewhat reduced suggesting that the extra length introduced by the His-tag might change the properties of the portal protein. To test that possibility, we purified a portal variant based on naturally occurring portal mutant in which 49 amino acid residues at the C-terminus (designated P22dif-his) are cleaved off and to which a C-terminal His-tag was appended. It was previously shown that the C-terminal 50 amino acids of the portal protein are dispensable and can be removed without significantly affecting viability of the phage (Bazinet et al., 1990). Indeed, P22 phage strains prepared with P22dif-his portal displayed replication efficiency similar to that of wild type (data not shown). Therefore, we studied the properties of both His-tagged full length P22fl-his and truncated P22dif-his portal proteins.

2.2. Mass spectrometric analysis of portal proteins

Electrospray ionization of proteins or protein complexes results in the formation of gaseous ions with a range of different charges (Figs. 3–6). Mass analyzers detect mass-to-charge ratios (m/z). Therefore, the resulting mass spectrum of a given species displays a cluster of peaks representing the molecule of interest carrying different number of charges. The m/z of a protein with mass m and charge z is given by:

$$m/z = \frac{m + (z \times 1.007)}{z}$$

where 1.007 is a mass of proton. By taking the m/z values of two adjacent ion signals of the same species, that have a relationship given by $z_2 = z_1 + 1$, two equations with two unknown are extracted from which the charge and thus the mass can be determined. In practice, an algorithm is used to average the mass and charge measurement over all ion signal present for a certain species.

Because of the potential for a native protein to retain solvent or even ligands in the gas phase accurate determinations of subunit masses are usually made in the denaturated form. Electrospray mass spectrometry of portal protein subunits denatured by passage through and elution from a reverse phase column has produced the following

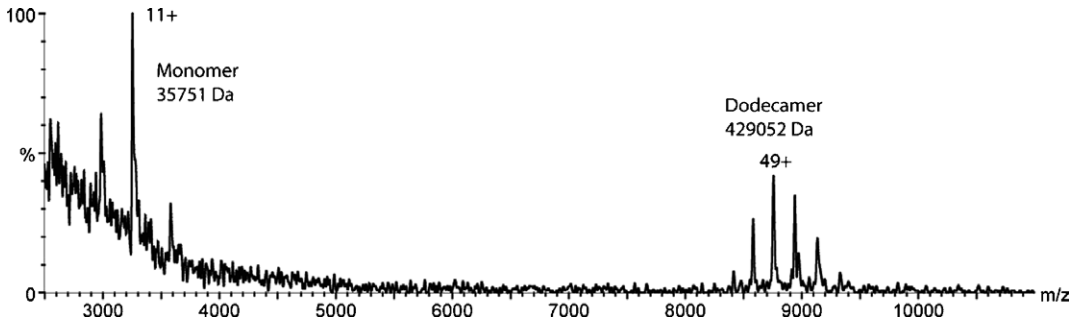

Fig. 3. Electrospray ionization mass spectra of the reconstituted Phi-29 portal sprayed from 50 mM aqueous ammonium acetate. The charge distribution centered around m/z 9000 originates from the dodecamer. Additionally, around m/z 3000 some weaker signals are observed from the Phi-29 monomer signals. These spectra indicate that the Phi-29 portals are present at a homogeneous stoichiometry of 12.

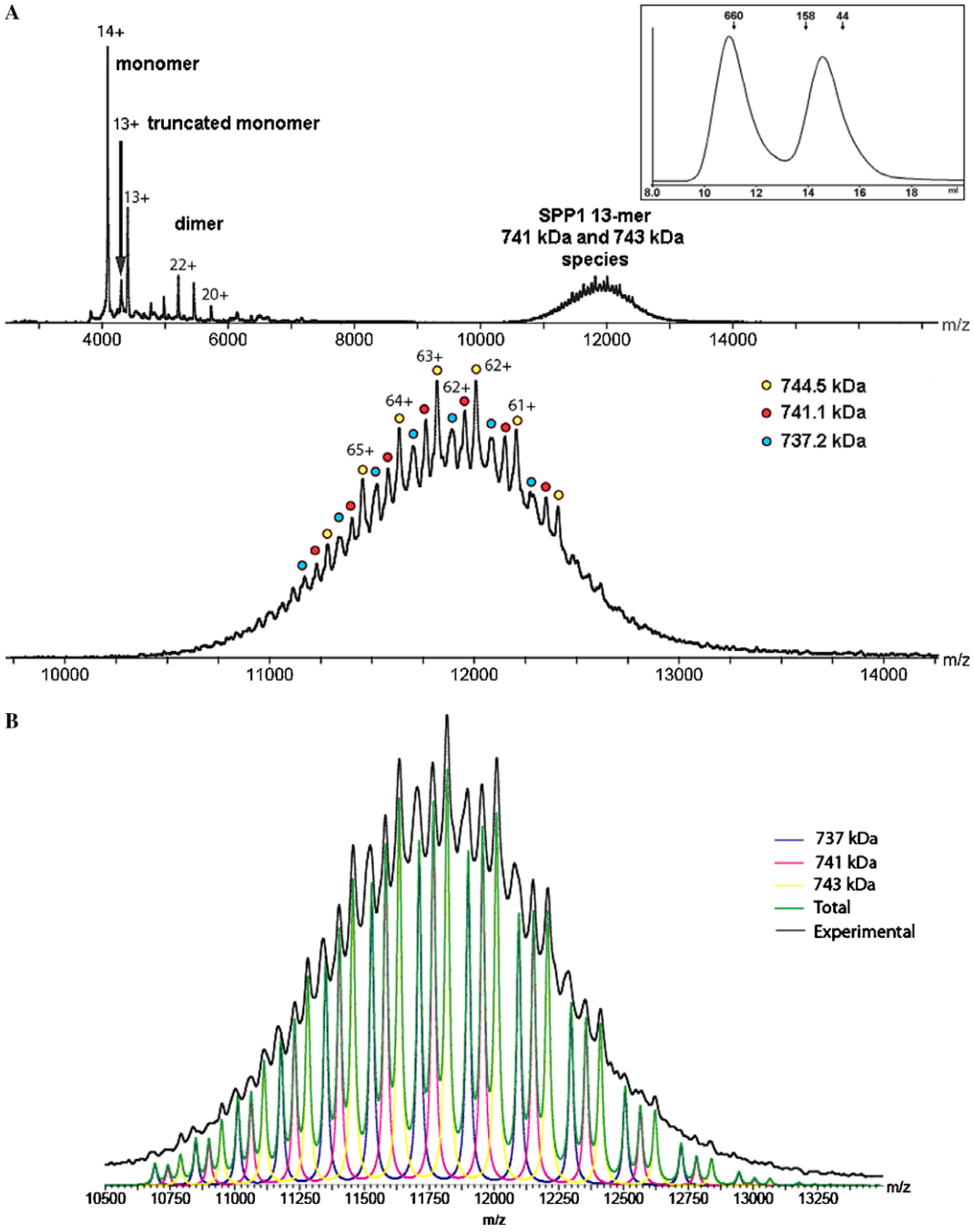

Fig. 4. Electrospray ionization mass spectra of the reconstituted SPP1 portal sprayed from 50 mM aqueous ammonium acetate. (A) The full-range ESI-MS spectrum. The most intense peaks in the mass spectra are of monomer ions (at m/z 4000) and ions originating from the tridecamer at m/z 12 000. The inset shows the size exclusion chromatogram of the SPP1 connector in the 50 mM ammonium acetate buffer used for spraying the protein (ammonium acetate), which is different from the SEC obtained in Fig. 2, and indicates some portal disassembly. Position of marker proteins peaks is indicated by arrows. Lower portion: zoomed-in view on the 13-mer signals centered around m/z 12 000. (B) Simulated (curve-fitted) mass spectrum of the spectrum shown in (B), revealing the presence of three tridecameric complexes in the reconstituted SPP1 portal sample. In this figure, the green line indicates the sum of the three simulated spectra of these three different 13-mers, showing excellent agreement with the experimental spectrum (in black). This analysis enabled to mass-determine and semi-quantify the abundance of the three species. The masses of the three assemblies were 743, 741 and 737 kDa, respectively in order of their relative abundance.

masses: 35 746.9 ± 1.8 Da, 57 201.7 ± 1.2 Da and 83 664.2 ± 2.8 Da, respectively, for the monomeric forms of Phi-29, SPP1, and P22 in reasonable agreement with the theoretical masses of 35 747.2, 57 201.3, and 83 666.6 Da.

2.3. Mass spectrometric analysis of portal assemblies

When sprayed from an aqueous ammonium acetate solution the portal assemblies are thought to retain their tertiary and quaternary structure. As a consequence the

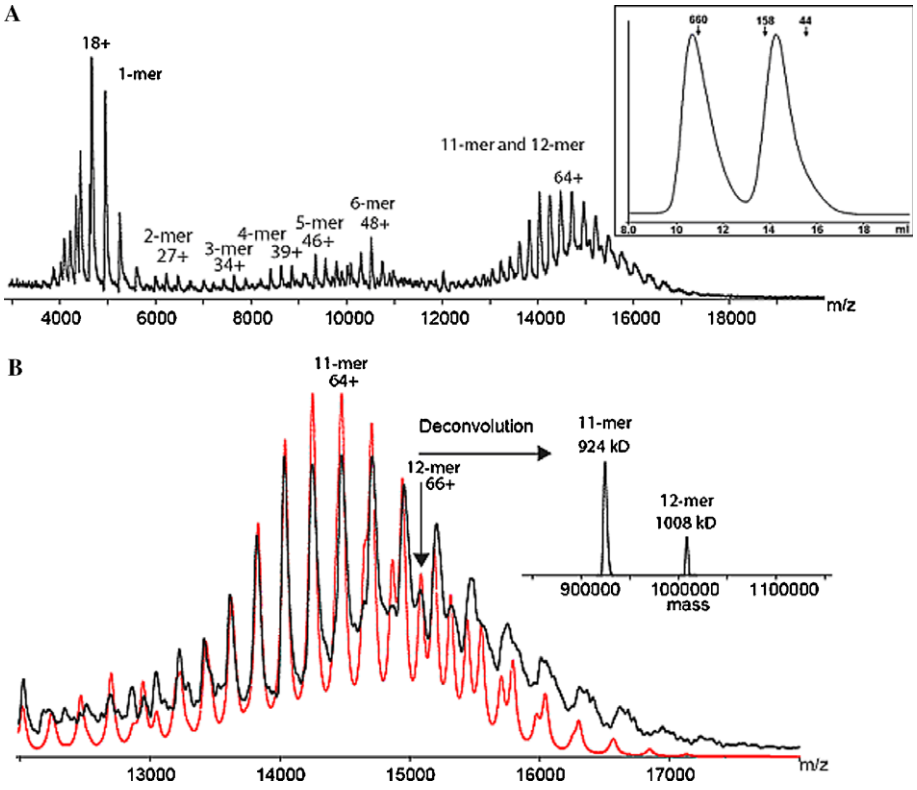

Fig. 5. Electrospray ionization mass spectra of the reconstituted P22fl-his portal sprayed from 50 mM aqueous ammonium acetate. (A) Full range ESI-MS spectrum. In the framed inset, the size exclusion chromatogram of the P22fl-his portal in 50 mM ammonium acetate buffer is shown, which again reveals the relative instability of the portals in this buffer. (B) Zoomed-in region focused on the undecamer and dodecamer P22fl-his portal assemblies shown in black. These spectra reveal that oligomeric complexes of the portal protein P22 are detected with several different stochiometries. Analysis and SOMMS simulation (in red in B) of these spectra reveal that monomeric, undecameric and dodecameric complexes are the most abundant species present, with their charge state distributions centered around 4500, 14 500 and 15 000 m/z, respectively. The simulated spectra (in red) in (B) are superimposed on the experimental spectrum and show consistency. The inset in (B) shows the zero-charge convolution of this spectrum, which indicates that the ratio between undecamers and dodecamers of the portal protein P22 is approximately 3:1.

species will attain considerably less charges in the ESI process as basic sites involved in higher-order structure formation and/or buried inside the structure of the assembly will no longer be accessible for protonation. The ions will still attain a range of charges through multiple protonation, but the number of charges will be dramatically reduced.

The analysis of macromolecular species by mass spectrometry requires some technical adjustments in addition to the use of a volatile buffer. While in general mass spectrometers are operated at low pressures to prevent collisions with residual gas molecules that may effect ion trajectories and thus lead to reduction of the mass-resolution, obtaining high transmission and thus good ion signals for macromolecular complexes requires somewhat elevated pressures in several regions of the mass spectrometer to slow down heavy ions and dampen their motions in the direction orthogonal to the ion transfer path by so called collisional cooling or collisional damping (Krutchinsky et al., 1998; Tahallah et al., 2001).

The proper desolvation of the proteins or protein complex ions is generally achieved by increasing the voltages used to guide the ions through the first stages of the mass spectrometer (in our instrument we typically increase the so-called cone-voltage, which is the potential difference between the sampling and extraction cone). Through numerous collisions with the residual gas in the first stages of the vacuum system solvent molecules evaporate from the protein species. Assemblies such as the portals studied here typically require quite high cone voltages to improve ion transmission and desolvation. However, a consequence of increasing the kinetic energy of the ions in this fashion is that they may also break up through collisional dissociation. Therefore, in the analysis of non-covalent complexes a balance must be found between proper desolvation, optimal ion transmission and prevention of dissociation of the complexes inside the mass spectrometer. For all portal assemblies examined here the highest attainable voltages resulted in the best desolvation and ion transmission and fortunately did not result in significant collisional dissociation of the assemblies in the source region of the mass spectrometer.

2.4. Phi-29 portal forms a dodecamer

Previous analysis of the symmetry of the connector of Phi-29 has proven controversial. There has been evidence for both 12-mers and 13-mers and the possibility that

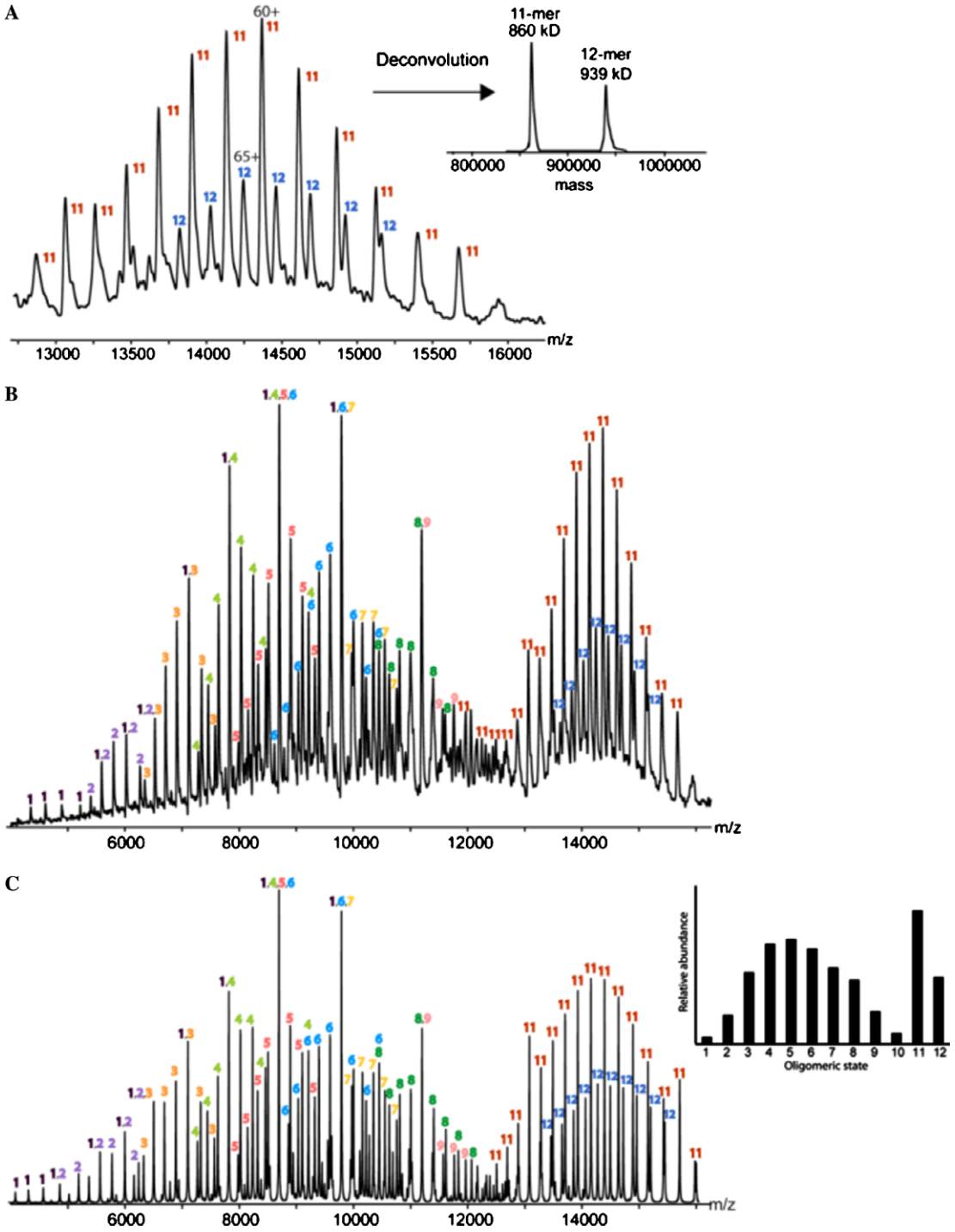

Fig. 6. Electrospray ionization mass spectra of the reconstituted P22dif-his portal sprayed from 50 mM aqueous ammonium acetate. (A) Zoomed-in part of the spectrum focusing on the undecameric and dodecameric ion signals. The inset in (A) shows the zero-charge convolution of this spectrum, which indicates that the ratio between undecamers and dodecamers of the portal protein P22 is approximately 2:1. (B) Whole range mass spectrum. Oligomeric assemblies with stochiometries varying from 1 to 8 and from 11 to 12 are present. (C) Analysis and SOMMS simulation of the whole ESI-MS spectrum. Peak position and intensity coincidence confirms correctness of assignment of different peaks. The inset shows the from the SOMMS simulation derived relative abundance of ions of different oligomeric states. The simulation confirms that the ratio between undecamers and dodecamers is approximately 2:1, and furthermore reveals that P22 is partly instable under the solution conditions used for electrospray, and dissociates primarily into species ranging from trimers to octamers. Interestingly, the nonamers and decamers are nearly absent in the ESI spectrum.

samples might be mixtures of both forms has been entertained (Tsuprun et al., 1994). In order to investigate this possibility, we performed mass spectrometry on Phi-29 connector. The mass spectrometric analysis of the Phi-29 portal was complicated by the fact that the protein seemed to precipitate readily in aqueous ammonium ace-

tate solutions at low ionic strength. In a separate series of solution experiments, we were able to reverse the precipitation by increasing the ionic strength suggesting that it is driven by electrostatic interactions. The crystal structure of the portal reveals that the narrow end (Guasch et al., 2002; Simpson et al., 2001) is negatively charged and at low ionic strength it may interact electrostatically with the positively charged wide end of the portal forming a linear aggregate. Apparent end-to-end aggregates have previously been observed by electron microscopy (Tsuprun et al., 1994). We were able to obtain a clear mass spectrum of the Phi-29 portal despite the complication due to precipitation (Fig. 3). The mass spectrum is dominated by a single charge state distribution centered at approximately 8700 m/z. Next to automatic peak annotation manual analysis was used to carefully determine the individual charge states of each peak, which indicated that these species had attained on average about 50 charges, and provided a measured mass of 429 052 ± 75 Da, which is in good agreement with the theoretical mass of a Phi-29 dodecamer of 428 966 Da. The fact that the measured mass is somewhat higher than the expected mass (about 0.025%) is a generally observed phenomenon in the analysis of macromolecular complexes by mass spectrometry and can be attributed to incomplete desolvation of the species (Sobott and Robinson, 2004). An additional cluster of peaks centered at approximately 3200 m/z corresponds to the folded monomer. The detection of the homogeneous Phi-29 dodecamer correlates well with the results obtained by crystallographic analysis of Phi-29 portal complexes (Badasso et al., 2000; Guasch et al., 1998; Simpson et al., 2001).

2.5. SPP1 forms tridecamers

When reconstituted SPP1 portal assemblies were electrosprayed a cluster of ion peaks centered at approximately 12 000 m/z could be detected, originating from SPP1 portal assemblies (Fig. 4A). In addition to these high m/z signals, signals from monomeric and dimeric SPP1 species were also present in the mass spectra. These species could be formed through disassembly in solution since it is known that SPP1 portals partially dissociate at low ionic strength in the absence of divalent ions (Jekow et al., 1999). To test this hypothesis, we analyzed the SPP1 portals by gel filtration chromatography using the aqueous ammonium acetate buffer used for mass spectrometry (Fig. 4A, framed inset). The resulting chromatogram revealed two distinct peaks, one of which corresponded to the oligomer as it eluted at the same position as in our previous experiment using the Tris buffer (see also Fig. 2). In aqueous ammonium acetate, one additional peak corresponding to the monomer elution time could be observed revealing that the portals are less stable in the aqueous ammonium acetate buffers used for mass spectrometry.

The ion signals centered at approximately 12 000 m/z were significantly more complex than those observed for the Phi-29 dodecameric portal (Fig. 4A and B). We were able to distinguish three separate partially overlapping charge distributions. However, for a more objective analysis of the mass spectra we used the recently introduced program SOMMS (van Breukelen et al., 2006), which is a curve fitting program that can be used to fit complicated mass spectra. Fig. 4B shows the electrospray mass spectrum of the SPP1 portals centered around m/z 12 000 together with the output of the curve fitting program. The curve-fitting confirmed our manual analysis of the spectrum and revealed unambiguously that the ion signals around m/z 12 000 indeed originated from three different species with molecular masses of 737 256 ± 93 Da and 741 121 ± 293 Da and 744 571 ± 66 Da, respectively. The 744 571 ± 66 Da species which generated the most abundant ion signal corresponded well to the mass of the tridecameric SPP1 portal (theoretical mass 743 616 Da). The two other species with masses of 737 256 ± 93 Da and 741 121 ± 293 Da evidently do not correspond to either a 12-mer (theoretical mass 686 415 Da) or a 14-mer (theoretical mass 800 818 Da). We hypothesize that since they have lower masses than the tridecamer they might originate from tridecamers containing truncated SPP1 subunits, still competent for assembly. More importantly, we conclude that reconstituted SPP1 portals form tridecamers exclusively.

2.6. Reconstituted P22 portal proteins are mixtures of undecamers and dodecamers

When we analyzed the reconstituted P22fl-his portal assemblies by mass spectrometry we obtained a highly resolved but relatively complex spectrum (Fig. 5). Again species originating from high molecular weight complexes were clearly present and centered around m/z 15 000. However, abundant ion signals were also observed at lower m/z ranges. These latter ions originated from monomeric and oligomeric P22fl-his portal assemblies, as indicated in Fig. 5. We hypothesized that the formation of these smaller assemblies may be due to the aqueous 50 mM ammonium acetate buffer used. Indeed, when reconstituted P22fl-his was analyzed in the ammonium acetate buffer by gel filtration chromatography lower molecular weight peak was observed (framed inset Fig. 5A), indicating that the P22fl-his complexes are also less stable in that solvent. Manual inspection and analysis of the spectrum using SOMMS (Fig. 5B) revealed that in the high m/z region, (around 15 000), the ions originated from two different species which could be assigned to the P22fl-his 11-mer (observed mass 923 698.6 Da, theoretical mass 920 332.6 Da), and the expected 12-mer (observed mass 1 008 211.6 Da, theoretical mass 1 003 999.2 Da). Curve fitting of the spectrum shown in Fig. 5B enabled us to semi-quantitatively assess the relative abundance of these two species as being 3:1, with the 11-mer being the dominant oligomer.

As we hypothesized that the previously described reduced burst size of P22 phage carrying P22fl-his portal

could arise from the fact that steric factors introduced by the His-tag might favor portal assembly towards an inactive 11-mer, we examined the P22dif-his, which has an essentially wild type burst. We reconstituted portal assemblies of this mutant using exactly the same experimental conditions, and analyzed these assemblies by electrospray mass spectrometry (Fig. 6). The electrospray ionization mass spectra of P22fl-his and P22dif-his portal (compare Figs. 5A and 6B) were very similar overall. However, the mass resolution of the spectra of P22dif-his turned out to be better making it possible to nearly base-line resolve the ions from the 11-mer (observed mass 861 937 ± 223 Da, theoretical mass 859 986 Da) and 12-mer (observed mass 939 789 ± 196 Da, theoretical mass 938 167 Da). We analyzed and simulated these spectra using SOMMS (Fig. 6C), and estimated that the ratio of 11-mer and 12-mer was ~2:1, with the 11-mer again being the dominant species. We therefore, obtain nearly identical results with two different constructs and conclude that the observation of 11-mer of P22fl-his is not a result of interference of His-tag with the polymerization. One might argue that the 11-mer could be formed through gas phase dissociation of the 12-mer inside the mass spectrometer. In this case, we would expect that the charge distribution of the 11-mer would be centered at a much lower value than the 12-mer because a subunit ejected during gas phase dissociation generally carries a disproportionate share of the initial charge (Felitsyn et al., 2001; Light-Wahl et al., 1994; Rostom et al., 1998; Schwartz et al., 1995; Versluis et al., 2001). Moreover, we observed that the ratio of 11-mer/12-mer was not influenced by a change in the cone voltage and it was not possible to break up either the P22fl-his or P22dif-his oligomers by collision induced dissociation even at highest collision voltage.

Finally, the fact that we observed virtually no ions attributable to decamer assemblies argues against gas-phase dissociation. It would be difficult to reconcile why such a break up process would stop at 11-mer and not proceed to decamer. Thus, we conclude that the 11-mer must be the most abundant species present in solution.

Unlike the Phi-29 and SPP1 portal assemblies, both P22fl-his and P22dif-his portals exhibit significant peaks that originated from smaller intermediate oligomeric assemblies. For P22dif-his the distributions of intensities of these smaller oligomers was determined by using the SOMMS simulated spectrum (Fig. 6C). Interestingly, this analysis reveals that the major intermediate assemblies range from tetramer to octamer, whereas nonamer and decamer are present in relatively low abundance. Although, there is no hard evidence, it may be suggested that these tetramer to octamer assemblies may act as intermediate structures in P22 portal assembly or disassembly.

2.7. Electron microscopy confirms the high abundance of undecameric P22 portal assemblies

To confirm that the 11-mers assemblies are indeed the predominant assemblies in reconstituted P22 we performed electron microscopy image analysis on P22fl-his. Portals were negatively stained with uranyl acetate and several hundred micrographs were taken. From these micrographs, top-view portals were selected manually and reference-free class averages were created. The resulting classes were unambiguously 11-fold (Fig. 7A), a feature further evidenced by application of 11-fold symmetry to the centered image (Fig. 7B). While it was not possible to accurately deduce the abundance of 11-fold relative to 12-fold, it was very clear a majority of the particles were in fact closed 11-mer rings, which is consistent with the mass spec data.

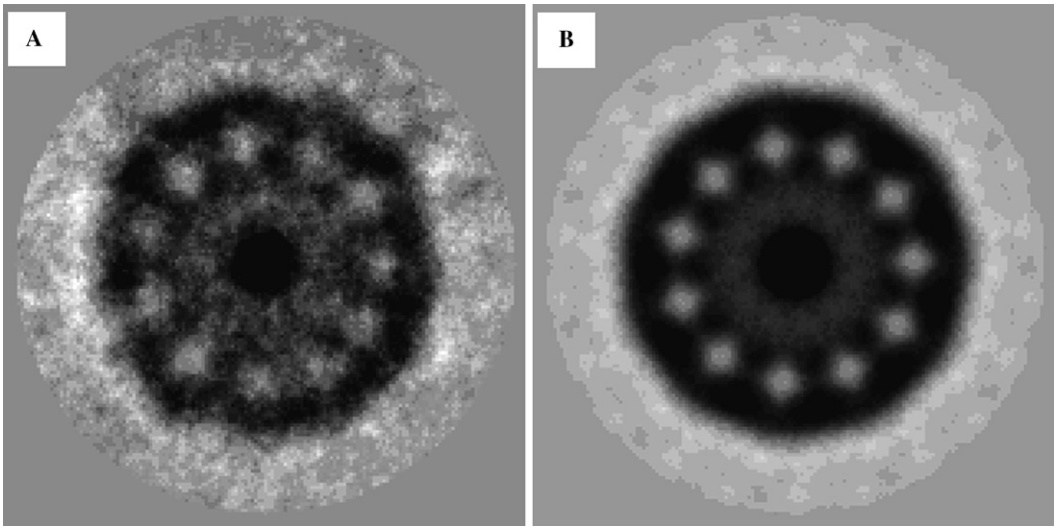

Fig. 7. Electron microscopy of P22 portal. (A) Result of reference-free class averaging. This view was achieved by performing reference-free alignment of the particles without assuming any symmetry inherent in the particle. (B) Application of 11-fold symmetry to the reference-free class average display, unambiguously, that the portal is 11-fold.

3. Discussion and conclusion

3.1. The stoichiometry and molecular mass of reconstituted Phi-29, SPP1 and P22 portals may be determined with high accuracy by macromolecular mass spectrometry

Our present data reveal that mass spectrometry is a valuable tool in structural biology as it allows determination of stochiometries and masses of macromolecules with an accuracy that is unmatched when compared to more conventional techniques such as gel-filtration chromatography or analytical centrifugation. For Phi-29, SPP1 and P22dif-his portal assemblies the deviations of the measured masses from the theoretical ones were 0.02%, 0.13% and 0.15%, respectively. More importantly, the high resolving power of this mass spectrometry-based approach allows detection of complex mixtures even when minor sub-populations of oligomers differing by only one subunit are present. As the structural analysis by electron microscopy, X-ray crystallography or NMR spectroscopy is severely hampered when the sample of interest consists out of a mixture of assemblies, macromolecular mass spectrometry may be used as pre-screening method to asses structural homogeneity.

3.2. The dominant form of reconstituted P22 portal assembly is an undecamer

The analysis of the bacteriophage Phi-29 and SPP1 portals by macromolecular mass spectrometry clearly revealed that these reconstituted portal assemblies form solely dodecameric and tridecameric species, respectively, in agreement with previously reported data on these portals (Guasch et al., 1998; Orlova et al., 1999; Simpson et al., 2000; van Heel et al., 1996). However, our data on the P22 portal assemblies were less expected. We previously reported that the P22 portal protein polymerized *in vitro* into dodecamers (Cingolani et al., 2002; Tang et al., 2005). Our present macromolecular mass spectrometry data indicate that while dodecamers are present in the population, the majority of the portal assemblies are undecamers. This interesting observation was confirmed by electron microscopy, whereby image analysis revealed unambiguously the high abundance of ring-like structures with 11-fold symmetry (Fig. 7). The previously reported crystallographic data (Cingolani et al., 2002) is, however, also quite unambiguous and therefore, we believe that the crystallization conditions either resulted in the selection and crystallization of the dodecamer subpopulation or facilitated the conversion of undecamers to dodecamers. In the case of electron microscopy the resulting image reconstructions were 12-fold averaged based on the results of the crystallographic studies. Upon revisiting the original data, processing without assumption of any given symmetry revealed that the portals were in fact overwhelmingly 11-fold, and that the crystallographic studies may have been preferentially selecting for 12-fold oligomers.

3.3. Comparing in vitro with in situ portal assembly

Bacteriophage portals studied *in situ*, i.e., when located in the bacteriophage capsid, exhibit exclusively 12-fold symmetry (Lurz et al., 2001; Carrascosa et al., 1985; Donate et al., 1988; Kochan et al., 1984; Jiang et al., 2006; Tang et al., 2005; Chang et al., 2006; Agirrezabala et al., 2005a; Lander et al., 2006). However, the present findings that the *in vitro* reconstitution of portals may lead to different stochiometries are also not unprecedented. For example, it was previously shown that the natural curvature of partially assembled SPP1 portal rings is neither precisely that of the dodecamer found *in situ* or the tridecamer found *in vitro* but lies closer to the tetradecameric form suggesting that the *in situ* form of the portal might be strained (van Heel et al., 1996). As the portal is a component of the packaging motor, this enforced strain may have a biological function. Portals assembled *in vitro* may represent the more "relaxed" oligomeric state. Moreover, high fidelity assembly of a portal dodecamer *in situ*, whether strained or not, may require host or viral factors to chaperone portal assembly. Head completion proteins (like gp15 of SPP1 (Orlova et al., 2003)) are not likely candidates for directing assembly of the portal into a dodecamer as they are attached to the capsid only after the genome packaging is complete, i.e., after the portal has already been assembled and incorporated into the prohead.

Interestingly, recent asymmetric reconstructions of electron microscopy images of mature infectious bacteriophages epsilon15, T7 and P22 have demonstrated that there is protein density inside the virions capping the portal complex (Agirrezabala et al., 2005a; Chang et al., 2006; Jiang et al., 2006; Lander et al., 2006). In the case of P22, this density likely corresponds to gp7, gp16 and gp20—three of the so called ejection proteins (Israel, 1977). It is tempting to speculate that one, or several, of these proteins might co-assemble with the portal prior to its incorporation into the prohead and, thus, aid in directing assembly towards the dodecamer.

Summarizing, we have demonstrated that macromolecular mass spectrometry may be used to analyze the stoichiometry of bacteriophage portals, reconstituted *in vitro*. The mass resolving power of the technique allows the semi-quantitative analysis of oligomeric mixtures, even when their masses exceed a million Da. Following this proof of principal, we conclude that this technique has great potential in the analysis of macromolecular assemblies, and more particular in various aspects of virus assembly. It may be of interest to study the assembly of portal complexes in the presence of the aforementioned ejection proteins or tail–spike associated proteins, but even the analysis of whole intact smaller viruses will be possible.

4. Materials and methods

4.1. Portal protein expression and purification

Phi-29 portal protein was expressed in *E. coli* from the plasmid pPLc28D. The protein was purified as described in (Ibanez et al., 1984) with several modifications. The cell pellets were resuspended in buffer containing 0.3 M NaCl, 50 mM Tris, pH 7.7, and treated with 200 ng/ml lysozyme and 50 ng/ml DNase & RNase on ice. The protein was purified with HiTrap SP HP column using a NaCl gradient from 0.3 to 1 M in 50 mM Tris–HCl, pH 7.7. The protein eluted at around 0.75 M NaCl and was dialyzed against buffer with 0.3 M NaCl and 50 mM Tris, pH 7.7. The second step of purification was carried out with HiTrap Q HP column using a NaCl gradient from 0.3 to 1 M in 50 mM Tris–HCl, pH 7.7. The protein eluted at around 0.43 M NaCl.

Cloning and purification of SPP1 connector protein has been published previously (Jekow et al., 1999). Briefly, *E. coli* BL-21 (DE3) (pLysS) transformed with plasmid containing full length, wild type gene 6 of SPP1 was grown at 37 °C in LB media containing 100 μg/ml ampicillin and 30 μg/ml chloramphenicol to $OD_{600} = 0.6$. Protein overexpression was induced by 2 mM IPTG for 2 h. Cells were harvested by centrifugation at 10 000*g* for 10 min, resuspended in 20 mM Tris, pH 7.6, 10 mM $MgCl_2$, 50 mM NaCl, 2 μg/ml DNAse and lysed by single passage through French press. Cell lysate was centrifuged at 15 000*g* for 40 min and the supernatant was precipitated by 50% ammonium sulfate. The precipitate was dissolved in 20 mM Tris, 20 mM $MgCl_2$, 50 mM NaCl, pH 7.6, and dialyzed against the same buffer overnight at 4 °C. The dialysate was subjected to anion exchange chromatography on 5 ml HiTrap Q Sepharose column (Amersham). Proteins were eluted by applying linear gradient of 10 column volumes of buffer containing 1 M NaCl. Fractions containing protein of interest were aliquoted and stored at −80 °C.

Cloning and purification of full length His-tagged P22 connector protein (termedP22fl-his) has been described previously (Moore and Prevelige, 2001). Additionally, a truncated version of P22 lacking 49C-terminal amino acids (termed P22dif-his) was constructed, expressed and purified. Histidine-tagged P22fl-his and P22dif-his connector proteins were expressed in *E. coli* BL-21 (Novagen) and purified by IMAC using 5 ml HiTrap chelating Sepharose column (Amersham) as described in (Moore and Prevelige, 2001). Eluate form HiTrap column was dialyzed overnight against 50 mM Tris, 50 mM NaCl, 2 mM DTT, 10 mM $CaCl_2$ and subjected to anion exchange chromatography on 5 ml HiTrap Q Sepharose column (Amersham). Elution was achieved by applying linear gradient of 10 column volumes of buffer containing 1 M NaCl. Fractions containing the protein of interest were aliquoted and stored at −80 °C.

4.2. Estimation of purity and intactness of proteins

Purity of the expressed protein was initially checked by SDS–PAGE. Masses of the proteins were measured by electrospray ionization mass spectrometry (LCT, Micromass). This analysis revealed that the proteins were of good purity, although some proteolytic activity was observed, leading to protein fragments.

4.3. Chromatographic analysis of connectors

An AKTA system (Amersham) was used in all chromatographic procedures.

Separation of monomeric and oligomeric forms of P22fl-his and P22dif-his was achieved by anion exchange using 5 ml HiTrap Q Sepharose column (Amersham).

Tricorn10/30 column packed with Superdex G200 gel (Amersham) was used for size exclusion analysis of selected fractions of portal proteins in order to estimate their oligomeric state. The column was calibrated using Gel Filtration Standard calibration kit (Bio-Rad Laboratories) and Blue Dextrane for determination of the column's void volume.

4.4. Electron microscopy

P22 and SPP1 portal proteins were diluted to ~0.05–0.1 mg/ml and applied to a carbon-coated copper grid, blotted to remove excess material and stained for 10 s with 2% uranyl acetate in water. The grids were dried for at least 1 h and imaged on electron microscope (Hitachi instruments). For determination of rotational symmetries of P22fl-his portal 5 μl of sample at 0.1 mg/ml was dropped onto a parafilm sheet and allowed to settle for 1 min, at which point a glow-discharged, carbon coated grid was laid on the surface and adsorbed for 30 s. The grid was then removed from the drop of sample and 0.5 μl of 1% uranyl acetate was applied. The grid was then allowed to air dry. Negative stain data was collected on a Tecnai F20 (120 keV, Cs = 2) at 50 000× magnification using low-dose techniques at a focus range of 0.8–1.2 μm under focus. 732 4 k × 4 k images were collected on a Tietz CCD camera over 12 h using the LEGINON automated data collection software package. From this dataset 144 top-view portal particles were selected manually. The EMAN reconstruction package was used to center the particles and to determine the reference-free class averages and symmetries.

4.5. Preparation of portal solutions for electrospray ionization mass spectrometry

P22 and SPP1 portals were subjected to buffer exchange by gel filtration on Tricorn10/30 columns packed with Superdex G200 gel equilibrated with 50 mM aqueous ammonium acetate, pH 7.9 (P22fl-his and P22dif-his) or pH 7.6 (SPP1). High molecular weight fractions were

aliquoted and stored at −80 °C. Phi-29 portal was transferred into 50 mM ammonium acetate, pH 6.8, by sequential dilutions and concentrations on Ultrafree spin tubes (Millipore) with a 30 kDa cutoff. Overall dilution was around 10 000-fold to ensure that no or very little non-volatile salts were present in the final solution, as beneficial for electrospray ionization.

4.6. Mass spectrometry

Proteins were sprayed from solutions of ~5 μM (monomer). The mass spectrometric measurements were performed using an electrospray ionisation time-of-flight instruments LCT or modified Q-TOF1 (van den Heuvel et al., 2006) (Waters) equipped with a Z-spray nano-ESI source. Needles were made from borosilicate glass capillaries (Kwik-Fil, World Precision Instruments, Sarasota, FL) on a P-97 puller (Sutter Instruments, Novato, CA), coated with a thin gold layer by using an Edwards Scancoat (Edwards Laboratories, Milpitas, CA) six Pirani 501 sputter. To produce the intact high molecular mass ions *in vacuo*, the ions were subjected to collisional cooling by increasing source pressure to approximately 8.0 mbar (Tahallah et al., 2001). The needle voltage varied between 1100–1600 V and the sample cone voltage between 100–200 V. Source temperature was 60 °C. Pressure conditions were 8.0 times 10^{-2} mbar in the analyzer and 2.3×10^{-6} mbar in the ToF. All mass spectra were calibrated using cesium iodide cluster ions generated from an aqueous solution of cesium iodide (50 mg/ml).

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