Disulfide Linkage and Structure of Highly Stable Yeast-derived Virus-like Particles of Murine Polyomavirus

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VP1 is the major coat protein of murine polyomavirus and forms virus-like particles (VLPs) in vitro. VLPs consist of 72 pentameric VP1 subunits held together by a terminal clamp structure that is further stabilized by disulfide bonds and chelation of calcium ions. Yeast-derived VLPs (yVLPs) assemble intracellularly in vivo during recombinant protein production. These in vivo assembled yVLPs differ in several properties from VLPs assembled in vitro from bacterially produced pentamers. We found several intermolecular disulfide linkages in yVLPs involving 5 of the 6 cysteines of VP1 (Cys115–Cys20, Cys12–Cys16, Cys12/Cys16–Cys115, and Cys274–Cys274), indicating a highly coordinated disulfide network within the in vivo assembled particles involving the N-terminal region of VP1. Cryoelectron microscopy revealed structured termini not resolved in the published crystal structure of the bacterially expressed VLP that appear to clamp the pentameric subunits together. These structural features are probably the reason for the observed higher stability of in vivo assembled yVLPs compared with in vitro assembled bacterially expressed VLPs as monitored by increased thermal stability, higher resistance to trypsin cleavage, and a higher activation enthalpy of the disassembly reaction. This high stability is decreased following disassembly of yVLPs and subsequent in vitro reassembly, suggesting a role for cellular components in optimal assembly.
assembled in vivo ("yVLPs"), identify disulfide cross-links, and compare these results with those of conventionally in vitro assembled VLPs from VP1 produced in bacteria ("bVLPs").

**EXPERIMENTAL PROCEDURES**

**Purification of yVLPs from Kluyveromyces lactis—**VP1 (small plaque strain 16) was recombinantly expressed in the yeast strain *K. lactis* KD1/VP1 by high cell density fermentation, and intracellularly assembled VLPs were purified chromatographically as described previously (14). Briefly, VLPs were enriched from cell lysate by ammonium sulfate precipitation, followed by size exclusion (Superdex S200, GE Healthcare) and cation exchange chromatography (HiTrap HP SP, GE Healthcare) and a final size exclusion run (Superdex S500, GE Healthcare).

**In Vitro Assembled bVLPs from Bacterial VP1**—VP1 was recombinantly expressed in *Escherichia coli*, and pentameric VP1 was purified as described (8). Purified VP1 pentamers were assembled into VLPs by dialysis for 24 h at 25 °C against 0.4 M ammonium sulfate, 200 mM NaCl, 5% (v/v) glycerol, 0.5 mM GSH, 4.5 mM GSSG, 2 mM CaCl250 mM Tris, pH 7.4. Assembled VLPs were separated from non-VLPs by size exclusion chromatography (Superdex S200, GE Healthcare).

**Temperature Transitions of VLPs—**Temperature-induced denaturation of VLPs was monitored by fluorescence spectroscopy (excitation 280 nm, emission 340 nm). 600 nM VLPs in PBS (Jena Bioscience) were heated from 20 to 90 °C at a heating rate of 1 K min⁻¹ using a Fluoromax-3 (Horiba Jobin Yvon) equipped with a Peltier element (Wavelength Electronics, Lf-3751). Equilibration time was set to 1 min. Pentameric samples were generated by disassembly of VLPs with 20 mM TCEP and 10 mM EDTA at 20 °C for 2 h and measured immediately.

**Analytical Ultracentrifugation—**To discriminate between VLPs, higher oligomers and pentameric subunits samples were centrifuged at 20 °C, 40,000 rpm for pentamers, 10,000 rpm for VLPs in an An50Ti rotor. Scans were taken every 10 min.

**Disassembly of VLPs—**VLPs (0.1 mg/ml in 50 mM Tris, pH 7.4, 200 mM NaCl, 5% (v/v) glycerol) were disassembled by adding 200 mM DTT and 50 mM EDTA at different temperatures (4–40 °C). The disassembly reaction was monitored by a decrease in the light scattering signal at 400 nm using a Fluoromax-4 (Horiba Jobin Yvon). As the disassembly of yVLPs was carried out in the presence of an excess of DTT, disulfide reduction is not a rate-limiting step, so the kinetics should reflect structural rearrangements. The rate constant k of every reaction was determined by a monoexponential (bVLPs) or biphasic exponential (yVLPs) fit. Activation enthalpies and activation entropies were determined from an Eyring plot.

To analyze disassembly products directly by analytical ultracentrifugation or fluorescence spectroscopy, we also disassembled VLPs with 20 mM TCEP, 10 mM EDTA in 0.3 M Tris, pH 7.4, 5% (v/v) glycerol, 200 mM NaCl2 h at 20 °C. Note that using TCEP instead of DTT causes reduction of disulfides to become a rate-limiting step at the beginning of reaction and is therefore not suitable for kinetic studies.

**Limited Proteolysis with Trypsin—**VLPs (0.1 mg/ml) were treated with trypsin (Sigma) at a ratio of 5:1 (w/w) in 50 mM Tris, pH 7.4, 5% (v/v) glycerol, 200 mM NaCl, 20 mM DTT, and/or 10 mM EDTA. As trypsin activity is influenced by reducing and chelating agents, the disassembly conditions were modified accordingly. Samples were taken at several time points and immediately placed on ice, precipitated with 0.1% (w/v) sodium deoxycholate, 10% (w/v) TCA and analyzed with reducing SDS-PAGE. Bands corresponding to degradation products of VP1 were excised, digested in gel with chymotrypsin, and analyzed using nano-HPLC/nano-ESI-LTQ-Orbitrap-MS/MS (see below). Peptides were identified with the Proteome Discoverer 1.3 (ThermoFisher Scientific) using Mascot Server version 2.2. MS/MS experiments were reproduced at least twice. The disassembly process due to proteolysis was also monitored by measuring a decrease in the light scattering signal at 400 nm.

**Reassembly of VLPs—**1 mg/ml yeast VLPs were first disassembled in 300 mM Tris, pH 7.4, 200 mM NaCl, 5% (v/v) glycerol, 10 μg/ml RNase A (Peqlab) in the absence or presence of 10 mM EDTA and/or 20 mM TCEP for 2 h at 20 °C. Then the subunits were dialyzed against assembly buffer (0.8 M ammonium sulfate, 200 mM NaCl, 5% (v/v) glycerol, 0.5 mM GSH, 4.5 mM GSSG, 2 mM CaCl2, 50 mM Tris, pH 6.4) to allow reassembly. After 48 h, the buffer was changed to the standard buffer 50 mM Tris, pH 7.4, 200 mM NaCl, 5% (v/v) glycerol by dialysis overnight. Although these conditions (low/pH, high GSSG concentration) differ from those typically used for refolding processes, they are necessary to obtain reasonable VLP yields, as the assembly of VLPs is a highly cooperative process that is sensitive to pH and salt concentration (15). The disassembly and reassembly products were analyzed by analytical ultracentrifugation, transmission electron microscopy, temperature transitions, and disassembly studies.

**Disulfide Pattern of Yeast VLPs—**Intermolecular disulfide cross-links between VP1 monomers within the VLP were analyzed by LC/MS/MS on an Ultimate 3000 nano-HPLC system that was coupled on line to an LTQ-Orbitrap XL mass spectrometer (ThermoFisher Scientific) equipped with a nano-electrospray ionization source (Proxeon). 7 μg of VLPs in PBS, pH 6.0, were denatured with 8 M urea, optionally reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide, and digested in solution with trypsin and 1% (w/v) TCA. Data were analyzed using the Proteome Discoverer 1.3, Xcalibur 2.07 (ThermoFisher Scientific) and StavroX 2.05 (16). MS/MS experiments were reproduced at least twice.

**Cryo-EM and Data Analysis—**Purified VLPs (1 mg/ml in 50 mM Tris, 200 mM NaCl, 5% (v/v) glycerol, pH 7.4) were placed on a glow discharged holey carbon copper grid (Quantifoil). The grid was loaded into a Vitrobot (FEI) at room temperature and 80–100% humidity, blotted for 3 s with filter paper, and plunged into liquid ethane for cryoelectron microscopy. The cryo-EM images were recorded on Kodak SO-163 film at a magnification of ×38,000 using a Philips CM 200 transmission electron microscope at 200 kV with defocus values of 1–1.5 μm. Low dose conditions with an exposure of ~20 electrons/Å² per image were used. Images were digitized using the Nikon Super Coolscan 8000 ED densitometer (17). The images were scanned with a resolution of 6.35 μm per pixel and later averaged 2-fold in each direction, resulting in a resolution of 3.34 Å/pixel at the sample level. 2523 particles were boxed with the program EMAN Boxer (18) and used for the refinement. The defocus value of each micrograph was estimated by ctffind3, and phase...
flipping was applied for each particle by using the SPIDER software package (19). A geometrical shell was used as a starting model. The orientations and translational alignments of the particle images were refined iteratively against the model by projection matching in SPIDER. Icosahedral symmetry was imposed during the refinement. The progress of the iterative refinement was initially monitored by the improvement of the Fourier shell correlation curve, which changed little after around 12 cycles. Docking of the atomic model into EM densities and display were carried out using UCSF Chimera (20) or PyMOL (21) as a further validation. The resolution of the three-dimensional reconstitution was estimated based on the Fourier shell correlation curve, which cuts off to 0.5 at 20 Å.

RESULTS

Disulfides of VLPs—VP1 contains six cysteines (Fig. 1). Published x-ray structures of viral particles (Protein Data Bank code 1SID (1)) show intermolecular, intrapentameric disulfide bonds between cysteine 115 and cysteine 20 in all but the pentavalent "5-fold axis" pentamers. No other disulfide bonds were observed in the crystal structure, although the presence of additional disulfide bonds in the VLPs has been described (4) (also for homologous polyomavirus SV40 (22–24)), but cross-links have not been assigned. Cysteine connectivities within the VLPs were therefore analyzed using pepsin cleavage in combination with tandem mass spectrometry (see supplement 1 for representative MS/MS spectra). Comparison of the sequence coverage analysis of reduced and nonreduced VLPs (Table 1) allowed quantification of disulfide cross-links. We found that Cys116 is quantitatively linked with one of the N-terminal cysteines Cys12, Cys16, or Cys20 (within the presumed flexible N-terminal region of VP1) in both yVLPs and bVLPs. All VLPs also showed a nonquantitative disulfide bond between Cys274 and Cys774. Conversely, yVLPs possessed additional disulfide bonds between Cys16 and Cys16 and Cys12 and Cys20 that were not found in bVLPs, suggesting that VP1 subunits are highly connected by disulfides in yVLPs.

Thermal Stability of VLPs—Temperature-induced denaturation of VLPs revealed a significant difference in thermal stability for yVLPs compared with bVLPs and in vitro reassembled yVLPs. Whereas the thermal transition of bVLPs is characterized by a temperature midpoint of denaturation of $T_M = 50 \, ^\circ C$ under the given conditions, yVLPs showed a $T_M$ of 56 °C (Fig. 2). These values correspond to the stability of the VLPs and not just to that of the structured protein VP1, as pentameric VP1 generated by disassembly of either yVLPs or bVLPs exhibited a $T_M$ of 46 °C (Fig. 2A). In vitro reassembly of pentameric VP1 from yVLPs resulted in VLPs with a temperature midpoint of denaturation ($T_M = 50 \, ^\circ C$) lower than the original yVLPs and similar to that of bVLPs, demonstrating a fundamental difference between in vitro reassembled VLPs and in vivo assembled yVLPs.

To analyze the influence of disulfide bonds on thermal stability, we reduced yVLPs with TCEP, which led to complete reduction of all disulfide bonds as determined by nonreducing SDS-PAGE (data not shown). Reduced yVLPs showed only a slightly lower thermal stability ($T_M = 53 \, ^\circ C$) compared with original yVLPs, but still a much higher thermal stability compared with bVLPs (Fig. 2) and reassembled VLPs. Reoxidation of reduced yVLPs resulted in thermal stabilities comparable with original yVLPs, and tandem mass spectrometry once again revealed the yVLP-specific disulfide pattern in reoxidized yVLPs not found in bVLPs (Table 1). These data suggest that additional structural elements stabilize in vivo assembled

![FIGURE 1. Schematic view showing positions of cysteines in VP1. VP1 has six cysteines (spheres), three of which are located in the N-terminal region and three in the core domain; the position of the 5-fold axis relating monomers in each pentamer is shown. In the assembled capsid, the region between Cys70 and the core domain forms a clamps-like structure with the C-terminal residues of a neighboring monomer, which adopt different conformations depending on their position in the capsid (pentavalent (blue) and hexavalent (shades of green) pentamers).](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>TABLE 1: Disulfide cross-links of VLPs analyzed by MS/MS spectrometry</th>
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<tr>
<td>Disulfide cross-links of VLPs analyzed by MS/MS spectrometry</td>
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<tr>
<td>In vitro assembled bVLPs</td>
</tr>
<tr>
<td>Sequence coverage of reduced samples (% of 384 amino acids)</td>
</tr>
<tr>
<td>Sequence coverage of nonreduced samples (% of 384 amino acids)</td>
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<tr>
<td>Disulfide cross-links (present under nonreducing conditions only)</td>
</tr>
<tr>
<td>e.g. (Asp112–Thr117) to (Lys11–Leu30)</td>
</tr>
<tr>
<td>e.g. (Lys15–Leu40) to (Met1–Thr17)</td>
</tr>
<tr>
<td>e.g. (Gly8–Glu13) to (Cys20–Leu38)</td>
</tr>
<tr>
<td>e.g. (Leu265–Leu279) to (Leu265–Gly279)</td>
</tr>
<tr>
<td>Cys12–Cys20</td>
</tr>
<tr>
<td>e.g. (Glu4–Glu13) to (Cys20–Leu38)</td>
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<tr>
<td>e.g. (Cys12–Cys20)</td>
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<tr>
<td>e.g. (Lys15–Leu40) to (Met1–Thr17)</td>
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Murine Polyomavirus Stability
yVLPs compared with in vitro assembled VLPs and are maintained in yVLPs even after disulfide reduction, guiding correct disulfide bond formation upon reoxidation.

Disassembly of VLPs—Another possibility to gain insight into the stability of VLPs is to monitor the kinetics of the disassembly into their subunits, whereby the temperature dependence provides information about enthalpic and entropic contributions to this reaction.

The disassembly reaction was started by addition of DTT and EDTA and monitored by light scattering. The disassembly kinetics of bVLPs could be fitted to a comparably fast and monophasic reaction \( (k = 0.015 \text{ s}^{-1} \text{ at } 25 \degree C, \text{Fig. } 3B) \), whereas the disassembly reaction of yVLPs was much slower and exhibited at least two phases (Fig. 3A). As it is known that in vivo assembled VLPs contain nucleic acids (12, 25), here RNA, the disassembly was also monitored in the presence of a nuclease. Addition of an excess of RNase A to the disassembly reaction still resulted in a biphasic reaction, with rate constants similar to the reaction in the absence of RNase, but the amplitude of the slow phase decreased significantly (Table 2), and the amplitude of the fast phase increased in a compensatory fashion. Further analyses of the purified yVLPs indicated that the majority of particles contained nucleic acids of a size between 100 and 500 bases (average 300 bases) that could be digested completely by RNase A but not by DNase I, indicating incorporation of RNA into the yVLPs (data not shown). We therefore conclude that the slower yVLP disassembly reaction \( (k_2 = 0.0002 \text{ s}^{-1} \text{ at } 25 \degree C) \) is dominated by RNA-VLP interactions and thus does not describe a specific molecular process upon disassembly. However, the fast yVLP disassembly reaction corresponds to the disassembly of nucleic acid-free VLPs \( (k_1 = 0.0028 \text{ s}^{-1} \text{ at } 25 \degree C) \), which is still about 2 orders of magnitude slower than that of bVLPs or in vitro reassembled yVLPs.

In addition, the temperature dependence of the rate of disassembly reaction 1 \( (k_1, \text{Fig. } 4) \) indicated a very high activation enthalpy \( \Delta H^* = 103 \text{ kJ mol}^{-1} \) but a favorable activation entropy. This is quite different for the disassembly of bVLPs, which exhibited a strongly unfavorable activation entropy (Fig. 4 and Table 3). These differences in activation enthalpy \( \Delta H^* \) and activation entropy \( \Delta S^* \) indicate that the energy barrier of the disassembly reaction is governed at least in part by different molecular interactions in yVLPs and bVLPs.

To find out if the observed disulfide bond Cys\(^{274}\)–Cys\(^{274}\) contributes significantly to the stability of the VLPs, we compared wild type yVLPs with a cysteine variant C274S yVLPs and could detect no significant differences in thermal stability or disassembly kinetics (Table 3 and Fig. 5).

Tryptsin-induced Disassembly of VLPs—Commonly, disassembly of polyoma VLPs can be induced chemically by addition of chelating and reducing agents, such as EDTA and DTT. As described previously, bVLPs can also be disassembled into pentameric VP1 by addition of trypsin (14). In contrast, yVLPs proved resistant to trypsin both in the absence (14) and pres-

**TABLE 2**

<table>
<thead>
<tr>
<th>RNase A</th>
<th>( k_1 ) (s(^{-1}))(^{-1})</th>
<th>( k_2 ) (s(^{-1}))(^{-1})</th>
<th>Relative amplitude phase 1</th>
<th>Relative amplitude phase 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>With</td>
<td>1.9 ( \pm ) 0.057</td>
<td>0.3 ( \pm ) 0.009</td>
<td>0.51</td>
<td>0.49</td>
</tr>
<tr>
<td>Without</td>
<td>2.8 ( \pm ) 0.162</td>
<td>0.2 ( \pm ) 0.012</td>
<td>0.30</td>
<td>0.70</td>
</tr>
</tbody>
</table>
an ordered structure that is protected by disulfide bond formation.

**DISCUSSION**

Previous crystal structures of icosahedral polyoma VP1 virus-like particles (1), which consist of 12 pentavalent and 60 hexavalent pentameric subunits, have demonstrated that the capsids are stabilized in three ways as follows: through formation of a clamp-like structure from the N- and C-terminal peptides of individual pentamers, through Ca\(^{2+}\) binding, and through intermonomeric/intrapentameric disulfide bond formation between Cys\(^{20}\) and Cys\(^{115}\) (in hexavalent pentamers only). Despite the similarity in morphology of *in vitro* assembled bVLPs and yeast-derived yVLPs, there exist marked differences between the two preparations. Compared with bVLPs, yVLPs are more stable and rigid and disassemble at a slower rate, with a higher activation enthalpy and entropy. MS/MS data of yVLPs revealed the presence of disulfides (Cys\(^{12}\)–Cys\(^{20}\) and Cys\(^{16}\)–Cys\(^{16}\)) that presumably link the N termini of VP1 of different pentamers. Such disulfides were not observable in the previous crystal structure, which shows no electron density for the first 17 amino acids (1). Our data on bVLPs suggest the absence of these disulfides in *in vitro* assembled material. We further found evidence for the presence of disulfide bridges between Cys\(^{115}\) and either Cys\(^{12}\) or Cys\(^{16}\) in both bVLPs and yVLPs, as well as a nonquantitative Cys\(^{274}\)–Cys\(^{274}\) linkage. The cryo-EM reconstruction clearly shows additional highly ordered electron densities at the N-terminal regions of VP1 in the VLPs, with sufficient volume to encompass three to four VP1 N termini from neighboring pentamers, providing space for the formation of the above described intrapentameric disulfide bridges. Within the pentavalent pentamers, the highly conserved Cys\(^{115}\) (which is not cross-linked with Cys\(^{20}\) here) lies in close proximity to the N-terminal cysteines Cys\(^{12}\) and Cys\(^{16}\) of adjacent monomers, providing an explanation for the observed Cys\(^{115}\)–Cys\(^{12}\)/Cys\(^{16}\) disulfide. Because the distance between the “pentavalent” Cys\(^{20}\)/Cys\(^{115}\) thiol groups and those surrounding the 3-fold axes are ~40 Å in the crystal structure, the observed disulfide bridges (and densities) cannot simply be a covalent linkage connecting neighboring pentavalent and
hexavalent pentamers. Instead, we suppose that the N-terminal peptides intertwine to form defined globular domains with intermonomeric disulfide linkages.

Disulfide bonds are known to increase the thermal stability of viral capsids (26, 27). In this study, reduction of disulfide bonds only slightly decreased thermal stability of yVLPs compared with the original yVLPs. The additional interpentameric contacts represented by the foot-like structures are likely to be responsible for the higher stability in yVLPs, with the observed yVLP-specific disulfides providing additional rigidity to these structures, as disulfide reduction is required for trypsin-mediated yVLP disassembly; bVLPs, however (which exhibit no cross-links between N-terminal cysteines), are not protected against proteolysis in the absence of any reducing agent. However, disulfide bonds in bVLPs (and yVLPs) protect the VLPs against chemically induced disassembly under chelating conditions.

Alternatively, the cross-links may represent deviations from icosahedral symmetry within the VLPs. Although we deem this to be unlikely, the presence of the Cys274–Cys274 disulfide cannot be explained by either the published crystal structures or the EM structure presented here. This bond does not seem to affect the stability of VLPs, as we found no differences in thermal stability, disassembly, or disassembly activation barrier for the C274S variant VP1 yVLPs. Conversely, Cys274 is (along with Cys115) the most conserved cysteine in VP1 of all polyomavirus infectivity as has been shown previously for the related polyomavirus SV40 (28). The presence of this bond in substoichiometric quantities may therefore be an indication of significant local deviations from strict icosahedral symmetry.

The higher stability of VLPs recombinantly expressed in eukaryotes was already reported qualitatively for expression in Saccharomyces cerevisiae and insect cells (13, 29). Because yVLPs exhibit a stronger association of pentameric subunits compared with bVLPs, we assume that intertwining of the N-terminal regions, complemented by N-terminal disulfide cross-links, supplements the previously described clamp structure (30). The existence of N-terminal disulfide linkages has long been thought to cross-link different VP1 pentamers (4), and similar interpentameric N-terminal cysteine linkages have been discussed for VP1 in polyomavirus SV40 (22–24). Although we are unable to distinguish between cystine formation occurring in the course of in vivo yVLP assembly or extracellularly/post-purification, in vitro assembly of VP1 into bVLPs under well defined redox conditions shows that disulfide bond formation takes place only after VLP assembly.3 Furthermore, a VP1 variant in which all cysteines have

3 T. Klose and H. Lilie, unpublished data.
been substituted by serine is still able to form VLPs (31), clearly indicating that disulfides are not essential for the assembly process. However, it is conceivable that the very high local concentration of cysteines resulting from ordering of the N-terminal VP1 structure in the \textit{in vivo} assembles yVLPs could induce disulfide formation, even under intracellular reducing conditions. We observed that \textit{in vitro} reassembly of disassembled yVLPs results in particles of lower stability, similar to that of bVLPs, suggesting a role for host cell factors during intracellular assembly in yeast. Such cellular “assembly helpers” might include the following: (i) interaction of VP1 with cellular RNA and assembly of VLPs in the yeast nuclei (12), as the natural virus assembles in so-called “nuclear cell factories” (3); (ii) cellular chaperones or protein isomerases, which are known to interact with VP1 \textit{in vivo} (32) and can induce assembly of VLP into VLPs \textit{in vitro} (33); and (iii) cytoskeletal and nuclear matrix proteins, which have been shown to interact with VP1 during expression (12, 34). Even though the assembly of VLPs \textit{in vitro} is a spontaneous process (10) under appropriate conditions, cellular factors might control and direct this process to increase the efficacy of assembly and the structural homogeneity of viral capsids.

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