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Use of PEG to acquire highly soluble DNA-packaging enzyme gp16 of bacterial virus phi29 for stoichiometry quantification

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Abstract

All linear dsDNA viruses package their genome into a preformed procapsid via a ATP-driving motor involving two nonstructural enzymes or ATPase. This essential viral replication step has been investigated in the quest for new antiviral drugs. These DNA-packaging motors could be potential parts in nanotechnology. But both the low solubility and self-aggregation of all nonstructural enzymes have seriously hampered studies on these motors. Bacterial virus phi29 DNA-packaging motor has been well characterized. But the role of the nonstructural ATPase gp16 has not been well defined due to its hydrophobicity, low solubility, and self-aggregation. Here we report a novel approach to obtain affinity-purified, soluble, and highly active native gp16 with the aid of polyethylene glycol (PEG) or acetone. With several thousand-fold increase in specific activity in comparison to the traditional method, this unique approach has made the quantification of gp16 feasible. The basic functional unit of gp16 in solution was found to be a monomer, as determined by sedimentation and size exclusion chromatography. This result leads to a subsequent finding that the stoichiometry of gp16 for phi29 DNA-packaging was about 11 ± 2. These findings will facilitate the study on this novel motor that involves three pRNA dimers and a 12-subunit connector.

Keywords: Viral assembly; DNA-packaging; Packaging enzyme; ATPase; Molecular motor

1. Introduction

A common feature in linear dsDNA virus maturation is that its genomic DNA is translocated into a preformed procapsid with remarkable speed and packaged into near crystalline density (Black, 1989; Earnshaw and Casjens, 1980; Guo, 1994). A motor using ATP accomplishes this energetically unfavorable reaction (Bazinet and King, 1985; Guasch et al., 2002; Jimenez et al., 1986; Simpson et al., 2000). Most, if not all, of these motors involve two nonstructural components, with at least one of them exhibiting certain characteristics typical of ATPases (Guo et al., 1987b).

DNA-packaging as an essential viral replication step has been investigated in the quest for new antiviral drugs. Factors involved in DNA packaging have been used as model targets for inhibiting viral replication (Trottier et al., 1996; Zhang et al., 1995) and for developing drugs against CMV (Bogner, 2002). Research into the pRNA structure of the phi29 motor has led to the development of new methods to destroy the hepatitis B virus (Hoeprich et al., 2003). But studies on the mechanism of DNA-packaging and the design of drugs targeting the DNA-packaging protein have been seriously hampered by both the low solubility of these proteins after expression and the self-aggregation after purification. Since most of these proteins have been cloned, over-expressed, and purified, some interesting data on the ATPase activity assay of the DNA-packaging proteins have been reported (Grimes and Anderson, 1990; Guo et al., 1987b; Hamada et al., 1987; Hwang et al., 1996; Mitchell et al., 2002; Tomka and Catalano, 1993). However, progress in the stoichiometry quantification and mechanistic investigation of these proteins has been far from satisfactory. Not a single crystal structure of these proteins has been reported, and

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none of the stoichiometry of these proteins has been clearly demonstrated.

The DNA-packaging motor of bacterial virus phi29 is fascinating due to the involvement of six RNA (pRNA) subunits (Guo et al., 1987a, 1998; Hendrix, 1998; Zhang et al., 1998) coupled with the highest DNA-packaging efficiency available (Guo et al., 1986). This research on the phi29 DNA-packaging motor is inherently multi-faceted, and has therefore, provoked broad interest across a range of scientific communities, from virologists, to bacteriologists, to biochemists, to molecular biologists, and especially to scientists involved in biophysics and nanotechnology. The synthetic motor constructed in vitro using recombinant proteins and synthetic RNA is fully functional and can package up to 90% of the added DNA in vitro (Guo et al., 1986; Lee and Guo, 1995b). This phi29 DNA-packaging system has been frequently utilized by other scientists to conduct novel studies (Davenport, 2001; Guo et al., 1998; Simpson et al., 2000; Smith et al., 2001; Tao et al., 1998; Zhang et al., 1998). A measured force of over 50 pN suggests that the phi29 motor is one of the strongest bio-motors studied to date (Smith et al., 2001). The finding that the motor pRNA binds ATP (Shu and Guo, 2003) is an entirely new concept in RNA function, and it implies that ATPase activity might not be limited merely to proteins. Investigating the similarities between hexameric pRNA (Guo et al., 1998) and other hexameric DNA/RNA processing protein enzymes (Bowers et al., 2001; Burgess and Richardson, 2001; Ellison and Stillman, 2001; Geiselmann et al., 1993; Herendeen et al., 1992; Hingorani and O'Donnell, 2000; Leu and O'Donnell, 2001; Niedenzu et al., 2001; Sedman and Stenlund, 1998; West, 1996), including DNA-helicases and the transcription termination factor Rho, will facilitate a better understanding of DNA replication and RNA transcription. Studies on DNA-packaging could also reveal similarities to macromolecular transport across cell membranes, as is the case when mRNA and tRNA are transcribed in the nucleus and must pass through the nuclear membrane to reach the cytoplasm. Research into the function of pRNA dimers and trimers (Chen et al., 2000; Shu et al., 2003b) provides for a better understanding of the life cycle of retroviruses, which rely upon dimerization for replication (Paillart et al., 1996; Skripkin et al., 1994). These dimers and trimers, and the rest of the motor as a whole, have significant potential to be used as parts in biological nanotechnology.

The phi29 motor involves three essential components: the 12-subunit connector (Guasch et al., 2002; Ibarra et al., 2000; Simpson et al., 2000, 2001), the six-subunit pRNA hexamer (Guo et al., 1998; Hendrix, 1998; Trottier and Guo, 1997; Zhang et al., 1998), and a protein, gp16. The crystal structure of the connector has been solved (Guasch et al., 2002; Simpson et al., 2000), and three-dimensional models of pRNA monomer, dimer, hexamer and the connector/pRNA complex have been reported (Hoeprich and Guo, 2002). The hydrophobicity, low solubility, and self-aggregation of phi29 gp16 have hindered further refinement of our understanding of the mechanism of this DNA-packaging motor. Contradictory data regarding ATPase activity, binding location, and the stoichiometry of gp16 have been published (Grimes and Anderson, 1990; Guo et al., 1987b; Ibarra et al., 2001). All discrepancies have arisen due to the lack of a method to make gp16 completely soluble. Little progress has been made since the finding of ATPase activity and the overproduction and purification of the gene product of gp16 15 years ago (Guo et al., 1986). In that traditional method, gp16 was purified in a denatured condition, and active gp16 was obtained by dialysis against 4 mM KCl buffer for 40 min for renaturation. But the renatured gp16 aggregated again within 15 min after renaturation. More recently, it was reported that gp16 was made soluble in the cell by co-expression with groE (Ibarra et al., 2001). Indeed, active gp16 was purified through use of this over-expression system. But while co-expression with groE solved the problem of aggregation in the cell, it could not solve the problem of self-aggregation after purification. Both pRNA and gp16 have been found to bind ATP (Huang and Guo, 2003; Shu and Guo, 2003), but the role of gp16 in the phi29 DNA-packaging motor is still a mystery. There is great need for a procedure to make pure, soluble, active, and stable gp16, which would allow for further characterization of such a fascinating molecular motor.

This report describes the approaches used to obtain successfully pure, soluble, active and stable gp16 by the use of his-tag column and polyethylene glycol (PEG) or acetone. The specific activity of this native gp16 was increased 3400-fold when compared with the traditional method. This unique approach made the study of gp16 oligomerization and stoichiometry feasible. Since all linear dsDNA viruses use a similar mechanism for DNA-packaging, this method could be applied to the DNA-packaging proteins of other dsDNA viruses to increase their solubility, activity and stability.

2. Materials and methods

2.1. Cloning and expression of His-tagged gp16

Plasmid PET-32, which contains a His-tag coding sequence and a thioredoxin gene driven by a T7 promoter, was cut after these two genes and treated with T4 DNA polymerase in the presence of only dCTP. Plasmid pART7ED (Trottier and Guo, unpublished data) that contains a gp16 gene was used as a template to generate a 1031 bp PCR fragment with the following

primers: 5′GGTATTGAGGGTCGCATGGACAAG-AGTTTATTTTATAATCC3′ and 5′AGAGGAGAG-TTAGAGCCTTACTGTATACGCATCTTACG3′.

The PCR product was purified and then treated with T4 DNA polymerase in the presence of appropriate dGTP to generate the specific vector-compatible overhangs. This PCR fragment was mixed with the above vector for annealing. The plasmid was transferred into *Escherichia coli* BL21(DE3) or *E. coli* BL21(DE3)/pLys (Rosenberg et al., 1987) for expression.

2.2. Purification of His-tag gp16

2.2.1. Cell extract preparation

An overnight growth culture of *E. coli* BL21(DE3) containing the plasmid-coding gp16 was transferred by 50 times dilution to LB broth with 50 μg/ml ampicillin and incubated at 37 °C, 250 rpm for 2.5 h. After the addition of isopropylthiogalactopyranoside (IPTG) to a final concentration of 1 mM, the cell culture was incubated for an additional 2.5 h at 37 °C. *E. coli* cells were collected by centrifugation. The cell pellet was resuspended in binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris–HCl, pH 7.9) at a ratio of 40 ml binding buffer per liter of cell culture. The resuspended cells were lysed by passage through a French Pressure Cell. The lysate was centrifuged at 39 000 × *g* for 20 min at 4 °C to remove debris, and then the post-centrifugation supernatant was filtered through a 0.45-μm membrane.

2.2.2. Buffers used

(a) His-charging buffer: 50 mM $NiSO_4$. (b) Binding buffer: 5 mM imidazole, 500 mM NaCl, and 20 mM Tris–HCl, pH 7.9. (c) Washing buffer: 60 mM imidazole, 500 mM NaCl, and 20 mM Tris–HCl, pH 7.9. (d) Elution buffer: 1 M imidazole, 500 mM NaCl, and 20 mM Tris–HCl, pH 7.9. (e) Strip buffer: 100 mM EDTA, 500 mM NaCl, and 20 mM Tris–HCl, pH 7.9.

2.2.3. His-column chromatography

His-Bind resin was resuspended by swirling, and 2.0 ml of the slurry was added to a disposable polyprep column (Bio-Rad Laboratories, Inc.) fitted with a 23-gauge needle. The resin was allowed to pack under gravity flow. This provided a column volume of 1.4 ml and a flow rate of 14 ml/h. The column was washed successively with 12 ml of water, 20 ml of His-charging buffer, and 12 ml of binding buffer. 40 ml of cell extract was loaded and then washed with 10 ml of binding buffer. The column was then washed with 30 ml of washing buffer and eluted with 2 ml of elution buffer.

2.3. Preparation of other components needed for the assembly of the infectious phi29 virion in vitro

RNAs were prepared as described previously (Zhang et al., 1994). Briefly, DNA oligos were synthesized with the desired sequences and used to produce double-stranded DNA by PCR. The DNA products containing the T7 promoter were cloned into plasmids. RNA was synthesized with T7 RNA polymerase by run-off transcription and purified from a polyacrylamide gel. All mutant RNAs made by in vitro transcription were purified by excision from 8 M urea denaturing gels and quantified by both comparison with standard RNA in gels and UV absorbance with 1 OD_{260} equal to 40 μg/ml of pRNA. The sequences of both plasmids and PCR products were confirmed by DNA sequencing.

The purification of procapsids (Bjornsti et al., 1985; Vinuela et al., 1976), DNA-gp3 (Ortín et al., 1971), along with the preparation of the tail protein (gp9) (Garcia et al., 1983; Lee and Guo, 1995b), neck proteins (gp11, gp12) (Carrascosa et al., 1974), the morphogenetic factor (gp13) (Lee and Guo, 1995b), and the procedure for in vitro phi29 assembly have already been described (Lee and Guo, 1995b).

2.4. In vitro production of the infectious phi29 virion

Briefly, 100 ng of RNA in 1 μl RNase free H_2O was mixed with 8 μl of purified procapsids (10^{12} procapsids per ml) and then dialyzed on a 0.025 μm type VS filter membrane (Millipore) against TBE (2 mM EDTA, 89 mM Tris borate/pH 8.0) for 15 min at room temperature. The mixture was subsequently transferred for another dialysis against TMS (100 mM NaCl, 10 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.8) for an additional 30 min. The pRNA-enriched procapsids were then mixed with gp16, DNA-gp3 (10^9 molecules per ml), and ATP (1 mM) to complete the DNA-packaging reaction. Native His-tagged gp16 was added directly into the packaging reaction. Denatured gp16 (in 6 M guanidine chloride) was renatured by dialysis against KCl buffer (4 mM KCl, 10 mM Tris–HCl, pH 7.5) on ice for 40 min in advance (Guo et al., 1986). After 30 min incubation at room temperature, an excess amount of neck and tail proteins gp9, gp11, gp12 and gp13 were added to the DNA-packaging reactions to complete the assembly of infectious virions, which were assayed by standard plaque formation. One unit of gp16 activity is defined as producing 10^4 pfu/ml infectious viruses.

2.5. Assay for organic solvents on gp16 activity and definition and determination of gp16 activity

Organic solvents were added into TMS. The purified His-tagged gp16 was diluted with buffers containing specific organic solvents and assayed for activity with

the highly sensitive in vitro phi29 assembly system (Lee and Guo, 1994, 1995b).

One unit of gp16 is defined as the amount of gp16 activity necessary to produce 10^4 pfu/ml of infectious phi29 in a 20 μl packaging reaction containing 10^{12} procapsid per ml, 100 ng of pRNA with 10^9 copies of DNA-gp3 per ml, and 1 mM ATP with an excess amount of the neck and tail proteins gp9, gp11, gp12 and gp13.

2.6. Comparison of specific activity of gp16 obtained via the traditional GuCl/KCl denaturing/renaturing method and the His-tag PEG–acetone method

The gp16 denatured in 6 M guanidine chloride (GuCl) was diluted in the same denaturing buffer and renatured by dialysis against a KCl buffer (4 mM KCl, 10 mM Tris–HCl, pH 7.5) for 40 min before being applied to the highly sensitive in vitro phi29 assembly assay. Native, pure His-tagged gp16 was diluted in PEG buffer (see below) before phi29 assembly assay.

2.7. Glycerol gradient sedimentation

40 μl of His-tagged gp16 (150 μg/ml) or standard native protein markers were layered onto six parallel 5-ml linear 15–35% glycerol gradients in TMS buffer. After centrifuging at 35 000 rpm in an sw55 rotor at 4 °C for 22 h, the gradients were collected into 36 fractions from bottom to top before being applied to SDS gel or in vitro assembly assay.

2.8. Gel permeation chromatography on a Superdex 200 HR column

Purified His-tagged gp16 200 μl (200 μg/ml) was loaded on a Superdex 200 HR column (i.d. 10 mm × 30 cm) (Amersham) and eluted with 20 mM Tris–HCl (pH 8.0), 0.4 M NaCl at a flow rate of 0.5 ml/min. The relative elution volume of protein His-tagged gp16 was compared with that of standard markers.

2.9. Determination of stoichiometry by measuring the slope of log plots of concentration versus products

The method for stoichiometry determination has been described (Trottier and Guo, 1997; Chen et al., 1997; Lee and Guo, 1994, 1995a; Shu et al., 2003a). Concentration dependence was determined with the in vitro phi29 assembly system mentioned above in the presence of all components in optimal or excess concentration except for gp16 to be tested. The log of the concentration (x-axis, from low to high) was plotted versus the log of the yield (y-axis, pfu/ml). The unit length of the log scale of the x-axis and the y-axis were made equal in the plot in order to determine the angle between the x-axis and the concentration dependent curve. The value of the tangent (slope) for the angle of the log/log plots of concentration versus products for native His-tagged gp16 was obtained from the dilution curve. Stoichiometry of gp16 for phi29 DNA-packaging was predicted with the equation $Z = -1.58 + 2.4193T - 0.001746T^2$ [$T \in (0,1000)$, or $\angle\alpha \in (0,\ 89.9^\circ)$]. This equation was deduced from other components dilution curves with known stoichiometry (Shu et al., 2003a). Here Z is the stoichiometry, T is the tangent of the angle of the log/log plot curve of concentration versus products.

3. Results

3.1. Cloning, expression, and purification of His-tagged gp16

The gene coding for gp16 was fused with a 109-amino acid polypeptide of thioredoxin (Fig. 1A). Both a six-His-tag and a 15-amino acid S-tag were placed between thioredoxin and gp16. An S-tag can interact with ribonuclease S-protein, serving as a marker for gp16 identification. The native gene product of gp16 is 36.5 kDa, and the total size of the thioredoxin, His-tag, and S-tag is about 17.6 kDa. Thus, the size of the fused chimeric gp16 (His-tagged gp16) is 54 kDa (Fig. 1B).

The His-tagged gp16 was purified by one-step affinity column purification to homogeneity as shown in the SDS polyacrylamide gel (Fig. 1B). Restriction grade Factor Xa was used to remove the vector-encoded sequences. Since there was a secondary specific cleavage site in the gp16, it was almost impossible to obtain a satisfactory amount of target protein. We found that the fused protein was fully active and functioned in the same manner as gp16. Most of the work in this report involved this His-tagged gp16. Pure, denatured gp16 without modification, as reported previously (Guo et al., 1986), was used as control when necessary.

3.2. Incompetence of the His-tagged gp16 after purification

DNA-packaging and phi29 assembly assays in vitro revealed that the purified His-tagged gp16 had no activity after being eluted from the His-column. Activity was not detected with the proteins stored at −70, −20 or 4 °C, despite the fact that the cell lysate, before loading onto a His-column, exhibited biological activity for phi29 DNA-packaging. Further analysis showed that the loss of biological activity after the column procedure was not caused by any components of the binding, washing, eluting buffers, or by imidazole. The inactive His-tagged gp16 still showed only one 54-kDa band in SDS gel, so the loss of activity was not due to proteolysis, either. The logical conclusion is, therefore,

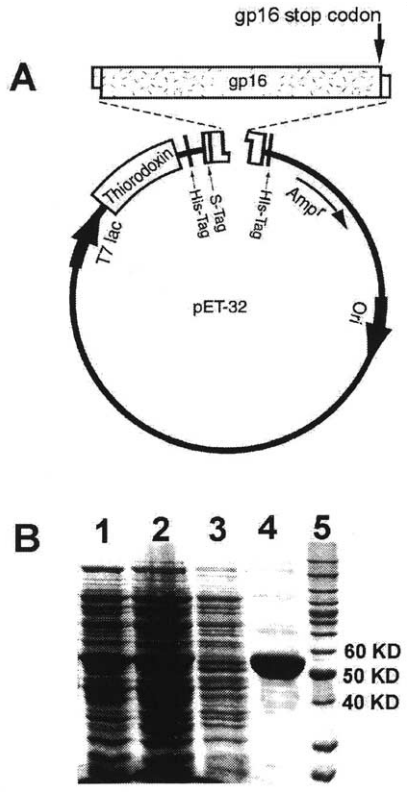

Fig. 1. Construction and purification of His-tagged gp16. (A) Map of plasmid harboring His-tagged gp16. (B) Affinity-purified His-tagged gp16 (10% SDS-PAGE). Lane 1–2: *E. coli BL21* (*DE3*) with plasmid that expressed His-tagged gp16. Lane 3: *E. coli BL21* (*DE3*) without plasmid. Lane 4: Purified His-tagged gp16. Lane 5: Protein markers.

that the loss of activity was due to His-tagged gp16 self-aggregation after His-column purification, since the formation of white-colored precipitates was observed and since aggregation is the same problem that all virus-packaging enzymes face. ATP with different concentrations (0.05–5 mM) was introduced from the cell lysate step in the protein purification process and was intended to help improve the solubility of protein, but it was not picked up.

3.3. Effect of solvents on phi29 assembly in vitro

The low solubility and self-aggregation of gp16 was demonstrated when gp16 was expressed in *E. coli* M5219 by a heat-inducible P_L promoter (Guo et al., 1986), and in *E. coli* BL21(DE3) by IPTG induction with a T7 promoter (Trottier and Guo, published results). It was found that the aggregation was due to the hydrophobicity of gp16. Before the question of whether a solvent can dissolve the hydrophobic gp16 and then restore activity was addressed, a variety of solvents were added to the highly sensitive in vitro phi29 assembly system. If a solvent inhibited phi29 assembly in vitro, it would not be a viable choice to dissolve His-tagged gp16.

Buffers containing acetone, chloroform, Tween-20, PEG-8000, *n*-octyl-β-D-glucoside (OG), ethanol, gelatin or acetonitrile were tested by adding varied concentrations of each reagent to the phi29 assembly mixture. It was found that phi29 assembly was inhibited by even a

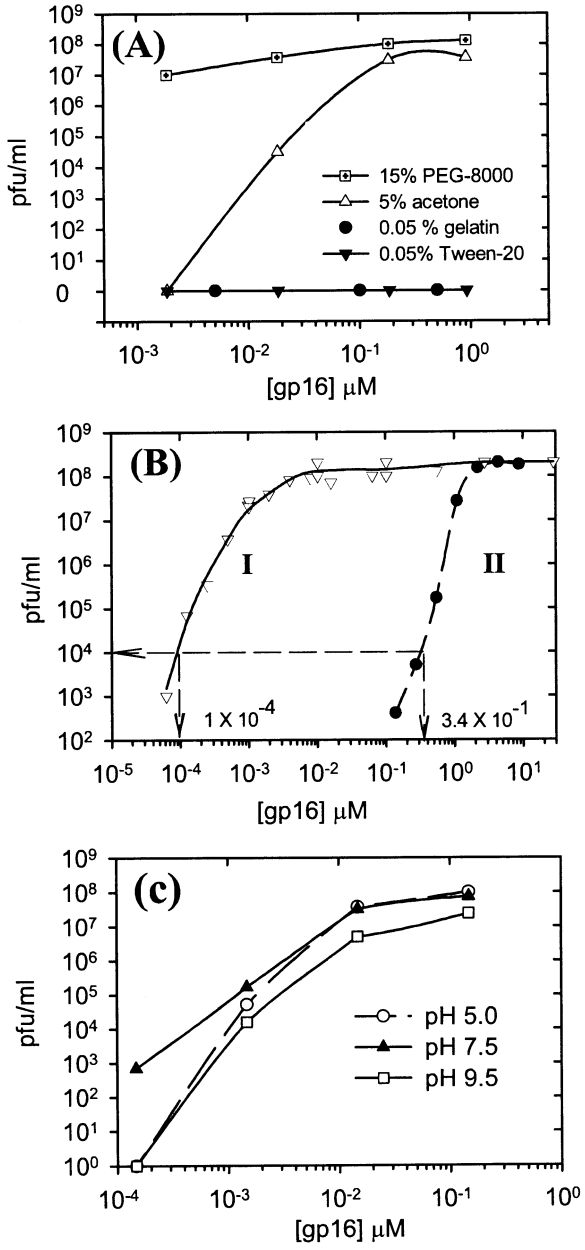

Fig. 2. Assay for gp16 activity. (A) Effect of solvents to restore His-tagged gp16 activity. (B) Specific activity of gp16 with PEG method (I) and tradition method (II). 1×10^{-4} μM of native gp16 in PEG buffer is comparable to 3.4×10^{4} μM of gp16 in the traditional GuCl–KCl method. This represents a 3400-fold ($3.4 \times 10^{-1}/1 \times 10^{-4}$ μM) increase in specific activity. (C) The effect of pH on PEG buffer. The gp16 denatured in 6 M guanidine chloride was diluted and dialyzed against the buffer (PEG buffer+4 mM KCl), with pH values ranging from 5.0 to 9.5.

tiny amount of acetonitrile, ethanol, OG or chloroform. These reagents were, therefore, excluded from further consideration. 5% Tween-20, 5% gelatin, 5% acetone, and 15% PEG each had no inhibitory effect on phi29 assembly. These solvents merited further examination.

3.4. Testing of organic solvents to restore His-tagged gp16 activity

The effect of individual organic solvents on His-tagged gp16 activity was further tested (Fig. 2A). PEG and acetone were the chemicals most efficient in the restoration of His-tagged gp16 activity. Gelatin and Tween-20 neither reduced nor enhanced His-tagged gp16 activity. The affinity-purified His-tagged gp16 maintained activity after being dissolved in PEG buffer (15% PEG 8000, 5% glycerin, 100 mM NaCl, 20 mM Tris–HCl, pH 7.8). 5% acetone can replace 15% PEG. The PEG buffer not only can be used for native His-tagged gp16, but also can be used to renature the gp16 denatured by guanidine chloride (GuCl) and enhance its activity as well. PEG-3350 also had a protective effect similar to that of PEG-8000 (data not shown).

3.5. With PEG or acetone, the specific activity of native gp16 increased 3400-fold

Traditionally, the overproduced, aggregated gp16 was dissolved in denaturing buffer containing 6 M GuCl. The in vitro assembly assay was applied directly after renaturing the protein by dialysis against 4 mM KCl buffer for 40 min. The renatured gp16 completely lost its activity within 15–20 min after renaturation. The definition of one activity unit of gp16 is 10^4 pfu/ml (half log activity) of infectious phi29 assembled in vitro. Specific activity is defined as the activity units of gp16 divided by the protein mass of gp16. The specific activity of His-tagged gp16 in PEG buffer was compared with the gp16 from the traditional GuCl–KCl method (Fig. 2B). In Fig. 3, 1×10^{-4} μM of native His-tagged gp16 in PEG buffer is comparable to 3.4×10^{-1} μM of gp16 used in the traditional GuCl–KCl method. This represents a 3400 times ($3.4 \times 10^{-1}/1 \times 10^{-4}$ μM) increase in the specific activity.

3.6. Shelf life affected by temperature and pH

The effects of temperature and pH on gp16 activity were investigated. Increasing the buffer pH from 5.0 to 9.5 did not improve gp16 activity in the PEG buffer (Fig. 2C). It was found that a temperature of −20 °C or lower was optimum for the preservation of gp16 activity; the lower the temperature, the better the shelf life became (Fig. 3).

3.7. Determination of the oligomerization status of soluble gp16 in PEG buffer by velocity sedimentation

The phi29 DNA-packaging motor contains 12 copies of gp10 (Guasch et al., 2002; Simpson et al., 2000) and

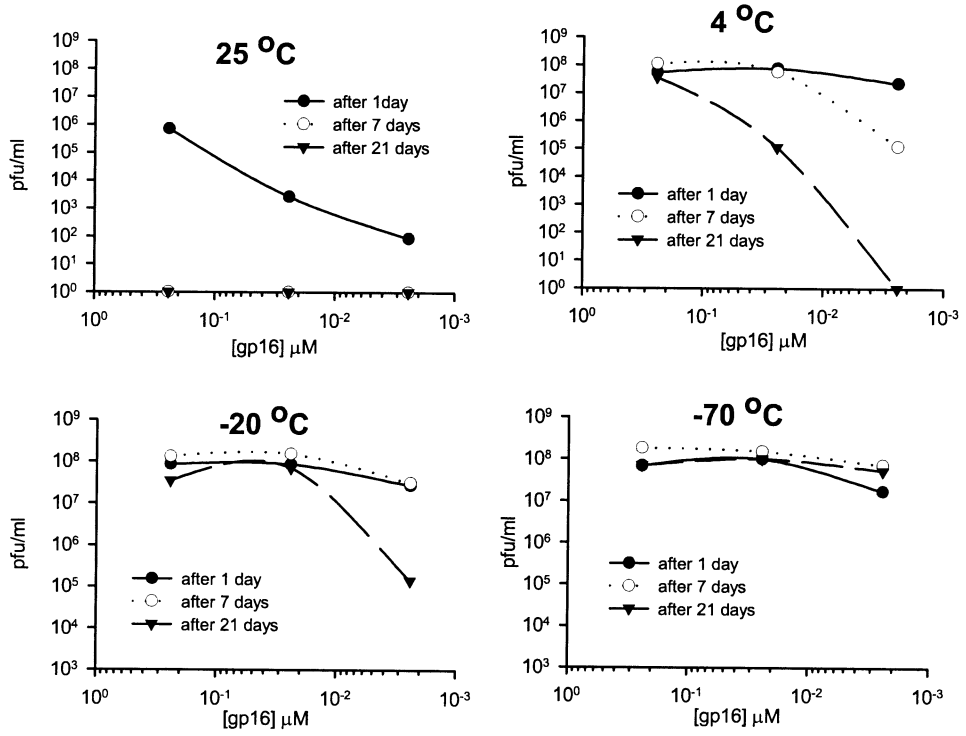

Fig. 3. Effect of storage time and temperature on the shelf life of His-tagged gp16. After purification, His-tagged gp16 was directly eluted into PEG buffer, stored at the indicated temperatures, and tested after 1 day, 1 and 3 weeks.

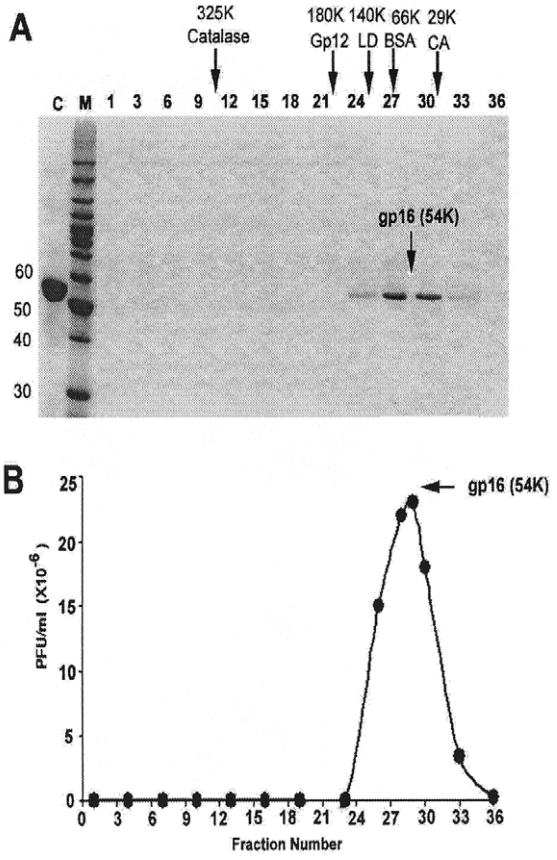

Fig. 4. Glycerol gradient sedimentation of His-tagged gp16. His-tagged gp16 and standard native protein markers were layered on the top of a 15–35% glycerol gradient individually. A total of 36 fractions were collected for each gradient. These fractions were subjected to (A) 10% SDS-polyacrylamide gel, and (B) the highly sensitive in vitro phi29 assembly assay. The number of fractions in the glycerol gradients correspond to that of activity assay. Standard native protein markers sedimented in a parallel gradient were: catalase, gp12, lactate dehydrogenase (LD), BSA, and carbonic anhydrase (CA). The position of size markers is indicated by arrows. C means His-tagged gp16 loaded into the gradient as positive control. M stands for protein molecular weight marker.

six copies of pRNA (Chen et al., 2000; Guo et al., 1998; Zhang et al., 1998) which appear as three dimers (Chen et al., 2000). Obviously, gp16 is involved in DNA-packaging, but its role and stoichiometry within the motor are still a mystery. Since pRNA hexamer is the active form for the phi29 DNA-packaging motor, elucidation of gp16's oligomerization status would facilitate studies on the role of gp16 in the phi29 DNA-packaging motor.

The size of the purified His-tagged gp16 or its oligomer complex in PEG buffer was first estimated by velocity sedimentation. SDS gel for each fraction of the gradient showed that the major peak of 54-kDa His-tagged gp16 is between that of 66 kDa (BSA) and 29 kDa (carbonic anhydrase) (Fig. 4A). This observation suggests that the majority of active His-tagged gp16 exists as a monomer in solution. An activity assay with the phi29 in vitro DNA-packaging system revealed that the peak of the activity overlapped with the peak in the sedimentation profile shown in SDS gel (Fig. 4).

3.8. Determination of the oligomerization status of soluble gp16 in PEG buffer by chromatography

To confirm further, the stoichiometry of His-tagged gp16 in solution, the purified His-tagged gp16 was analyzed by high-resolution gel permeation on a Superdex 200 HR column (Fig. 5). The elution volume for His-tagged gp16 was compared with that of standard markers. The major peak of the 54-kDa His-tagged gp16 appeared between 81 kDa (human transferrin) and 43 kDa (ovalbumin). This indicates that the His-tagged gp16 exists mainly as a monomer in solution, which agrees with the conclusion of the velocity sedimentation experiment.

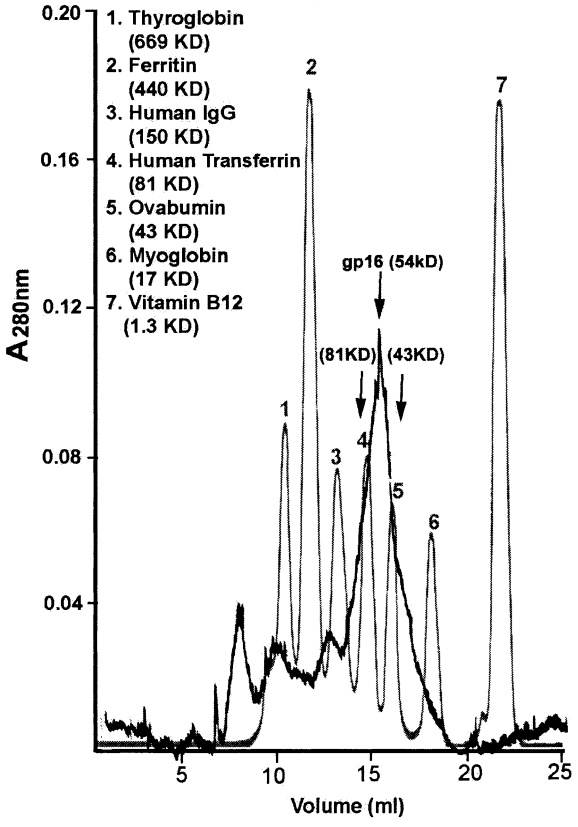

Fig. 5. Gel permeation chromatography on a Superdex 200 HR column. Purified His-tagged gp16 was loaded onto a Superdex 200 HR column (i.d. 10 mm × 30 cm) and eluted with 20 mM Tris–HCl (pH 8.0) and 0.4 M NaCl at a flow rate of 0.5 ml/min. The relative elution volume of His-tagged gp16 was compared with that of standard markers.

3.9. Stoichiometry determination for gp16 by slope of log/log plot curves for concentration versus yield

In the viral assembly study, it was found that the slope of the curve in the log/log plot of the concentration versus the product for each component is the intrinsic parameter reflecting the stoichiometry of the component (Chen et al., 1997; Lee and Guo, 1994, 1995a; Trottier and Guo, 1997). The larger the stoichiometry of the component, the more dramatic the influence of the component concentration (dilution factor) on the reaction will be. A slope of one indicates that one copy of the component is involved in the assembly of one virion, as is the case for genomic DNA or procapsid in phi29 assembly. A slope larger than 1 would indicate multiple-copy involvement. The dose–response curves of in vitro phi29 assembly versus concentration of various assembly components have been used as a method to approximate confirm the stoichiometry of pRNA, gp11, gp12, and gp9 (Chen et al., 1997; Lee and Guo, 1994, 1995a; Shu et al., 2003a; Trottier and Guo, 1997). An equation $Z = -1.58 + 2.4193T - 0.001746T^2$ [$T \in (0,1000)$, or $\angle\alpha \in (0,\ 89.9^\circ)$], has been deduced for stoichiometry determination (Shu et al., 2003a). This equation was used to predict the stoichiometry Z for native His-tagged gp16. The angle, $\angle\alpha$, of the curve representing native His-tagged gp16 was determined to be $79^\circ \pm 1$. The value of tangent (T) of $\angle 79^\circ$ is 5.2. From the equation $Z = -1.58 + 2.4193T - 0.001746T^2$, it was found that when T equals to 5.2, Z equals to 11. That is, the stoichiometry of gp16 for the packaging of one copy of phi29 DNA was about 11 ± 2 (Fig. 6).

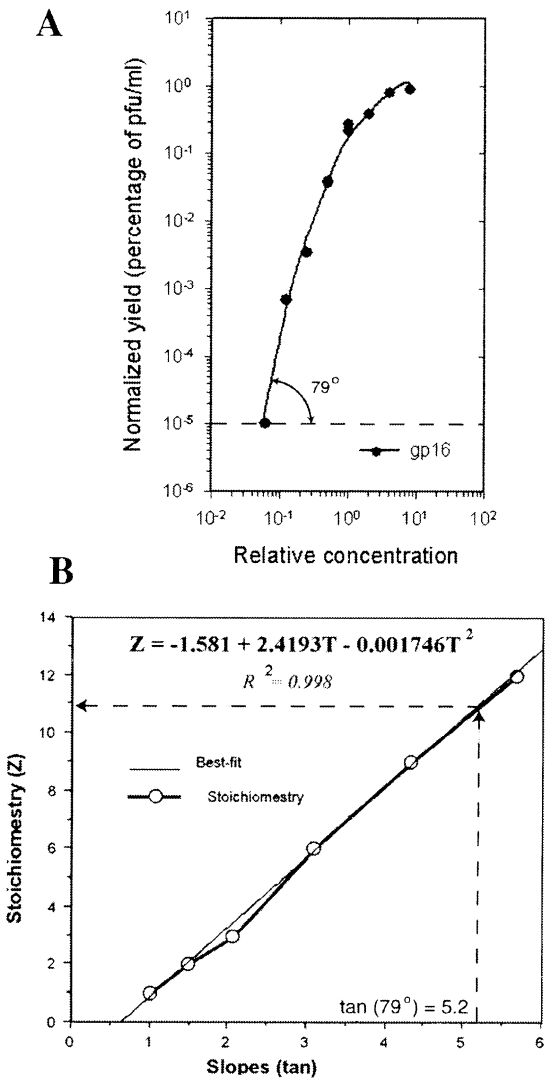

Fig. 6. Determination of stoichiometry of gp16 for phi29 DNA packaging. (A) Log/log plot of concentration vs. yield of virus production for His-tagged gp16. (B) Application of the standard curves and equation (Shu et al., 2003a) to find the stoichiometry of gp16. A stoichiometry (Y-axis) of 11 was found to be a correspondence of the tangent value (X-axis) of 5.2, which is the result of tan 79°.

4. Discussion

As noted earlier, research in phi29 DNA packaging has been seriously hampered by two special properties of gp16: its low solubility and tendency to self-aggregate. Several approaches have been employed in order to obtain soluble and active gp16. But until now, the published novel methods have only made gp16 soluble before purification; it re-aggregates and loses its activity minutes after purification. Like other DNA-packaging proteins of dsDNA viruses, gp16 is hydrophobic. This report shows that the idea to use an organic solvent such as acetone to keep gp16 soluble has become realistic. Although other organic solvents such as chloroform, OG, ethanol, and acetonitrile have been found ineffectual in this study, this difficulty might be due to the fact that these organic solvents inactivate other protein components involved in the assembly of infectious phi29 virus. This by no means indicates that other organic solvents could not help dissolve gp16 and then restore its activity. Whether or not other solvents can be used for assays that do not involve organic solvent-sensitive components remains to be investigated. For example, the ATPase assay does not involve such components. It would be worthwhile to test the feasibility of organic solvents in this area.

PEG has been found to enhance the efficiency of DNA-packaging (Louie and Serwer, 1994; Serwer et al., 1983). Interactions between DNA and cationic polymers, including poly-lysine and PEG, have attracted attention since polymers promote the formation of complexes by facilitating the oriented self-assembly of DNA with cationic-hydrophilic copolymers, which could enshroud the complex within a protective hydrophilic polymer corona. Cationic polymers appear to have the ability to regulate the physicochemical and

biological properties of polycation/DNA complexes. Polymers could, therefore, stimulate the packaging of nucleic acid in vitro (Toncheva et al., 1998; Yoshikawa, 1997). The phenomenon of PEG enhancement has been connected to the mechanism of viral DNA packaging (Louie and Serwer, 1994; Serwer et al., 1983), and our results indicate that PEG does indeed enhance the packaging efficiency of phi29. It is clear from this study that this enhancement is mainly due to the promotion of gp16 solubility. Whether the aforementioned hydrophilic polymer corona effect could facilitate the packaging of phi29 DNA remains to be investigated.

Previously, it was impossible to determine stoichiometry of gp16 due to its low solubility. With this native, pure His-tagged gp16 we can, for the first time, take advantage of the stoichiometry determination curve to predict the stoichiometry of gp16 in the DNA-packaging process is more than 6. A definite stoichiometry has not been determined to this point due to the possible reusability and recycling of this DNA-packaging enzyme. Potential areas for further inquiry include determining whether the gp16 is reusable and the mechanism of its function as an ATPase.

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