Published in final edited form as:

J Mol Biol. 2011 September 30; 412(4): 723–736. doi:10.1016/j.jmb.2011.07.045.

The Bacteriophage Lambda gpNu3 Scaffolding Protein Is an Intrinsically Disordered and Biologically Functional Procapsid Assembly Catalyst

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Abstract

Procapsid assembly is a process whereby hundreds of copies of a major capsid protein assemble into an icosahedral protein shell into which the viral genome is packaged. The essential features of procapsid assembly are conserved in both eukaryotic and prokaryotic complex double-stranded DNA viruses. Typically, a portal protein nucleates the co-polymerization of an internal scaffolding protein and the major capsid protein into an icosahedral capsid shell. The scaffolding proteins are essential to procapsid assembly. Here, we describe the solution-based biophysical and functional characterization of the bacteriophage lambda (λ) scaffolding protein gpNu3. The purified protein possesses significant α -helical structure and appears to be partially disordered. Thermally induced denaturation studies indicate that secondary structures are lost in a cooperative, apparent two-state transition ($T_m = 40.6 \pm 0.3$ °C) and that unfolding is, at least in part, reversible. Analysis of the purified protein by size-exclusion chromatography suggests that gpNu3 is highly asymmetric, which contributes to an abnormally large Stokes radius. The size-exclusion chromatography data further indicate that the protein self-associates in a concentration-dependent manner. This was confirmed by analytical ultracentrifugation studies, which reveal a monomer–dimer equilibrium ($K_{d,app} \sim 50$ μ M) and an asymmetric protein structure at biologically relevant concentrations. Purified gpNu3 promotes the polymerization of gpE, the λ major capsid protein, into virus-like particles that possess a native-like procapsid morphology. The relevance of this work with respect to procapsid assembly in the complex double-stranded DNA viruses is discussed.

Keywords

virus assembly; viral capsids; procapsid assembly; bacteriophage lambda; herpesvirus

Introduction

The essential features of virus development are conserved in the eukaryotic and prokaryotic viruses.^{1,2} For instance, common pathways for genome packaging are observed in the complex double-stranded DNA (dsDNA) viruses including adenovirus,³ the herpesviruses,^{4,5} and many bacteriophages.^{1,6} In these cases, a terminase motor packages the viral genome to near-crystalline density within a preformed “procapsid” shell.^{5–8} The

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2011.07.045

capsid is a robust structure that must withstand both environmental insult and the immense internal pressure generated by the tightly packaged DNA; improperly formed procapsids are unable to completely package the viral genome. Thus, fidelity in procapsid assembly is central to virus development and infectivity.

The polymerization of several hundred capsid protein monomers into an icosahedral shell could follow many assembly pathways.^{9,10} Yet, the capsid assembly pathways are strongly conserved in all of the complex dsDNA viruses. To prevent the formation of aberrant procapsids, bacteriophages such as λ , ϕ 29, P22, and SPP1 and the eukaryotic herpesviruses use internal scaffolding proteins to “chaperone” the major capsid protein into an icosahedral shell.^{11–14} *In vivo*, a doughnut-shaped portal structure typically nucleates the co-polymerization of scaffolding protein and major capsid protein into an immature procapsid. A single improper binding event could result in an aberrant procapsid structure and, thus, a noninfectious viral particle.^{5,8,15–18} The scaffolding protein is not a component of the mature shell; it exits the procapsid interior, either intact or degraded by a viral protease, around the time of DNA packaging.^{8,15–19} In several systems, the purified scaffolding protein can catalyze polymerization of the major capsid protein into a virus-like particle (VLP) *in vitro* in the absence of a nucleating portal structure.^{20–26}

Bacteriophage λ has been intensively studied. The wealth of genetic, biochemical, and biophysical data available make this an ideal system in which to study the mechanistic details of virus assembly.^{27–29} Assembly of the λ procapsid *in vivo* initiates with the formation of a gpB portal ring, which nucleates the co-polymerization of the gpNu3 scaffolding protein and gpE major capsid proteins (Fig. 1).^{29,30} This affords an immature procapsid shell composed of 415 copies of gpE and containing ~70–200 copies of gpNu3 within the interior.^{30,31} The gpB ring resides at a unique vertex of the icosahedron and serves as a portal through which DNA enters the capsid during packaging and exits during infection of the host cell.^{32,33} The gpC protease, which is also incorporated into the shell during assembly, then “matures” the procapsid by proteolysis (Fig. 1).^{19,32} This includes autoproteolysis, degradation of 20 N-terminal residues from roughly half of the portal proteins (gpB→pB*), and degradation and exit of the scaffolding protein from the procapsid interior.¹⁹ GpNu3 is required for efficient and accurate procapsid assembly but is not present in the final structure. Thus, it is presumed that gpNu3 acts as the scaffolding protein in procapsid assembly to prevent the formation of off-pathway intermediates.^{11–13,34}

It is commonly accepted that disorder and flexibility are fundamental characteristics of the viral scaffolding proteins.^{11–13} However, direct demonstration of these features using comprehensive, solution-based biophysical approaches has been made in only a few cases, most notably bacteriophages P22³⁵ and SPP1.³⁶ This presumption has over time become a *fait accompli*, and in many systems, these features are presumed *a priori*. For instance, primary sequence analysis indicates that the λ gpNu3 protein possesses low sequence complexity (26% Gly/Ala), has a preponderance of polar/charged residues (45%), and is devoid of aromatic amino acids (see Fig. 2a).³⁷ While these features are hallmarks of disordered protein structures,³⁸ this has not been experimentally demonstrated for gpNu3. Size-exclusion chromatography (SEC) and sucrose gradient centrifugation data suggest that the protein is a highly asymmetric monomer;³⁹ however, a rigorous biophysical and functional characterization of the λ gpNu3 scaffolding protein has not been performed. In this work, we describe a biochemical, biophysical, and structural interrogation of gpNu3 *in vitro*. We directly demonstrate that the purified protein is intrinsically disordered, assembles into a homodimer in a dynamic equilibrium, and is biologically active in promoting gpE assembly into VLPs with wild-type morphology.

Results

Purification and biochemical characterization of gpNu3

Purification of gpNu3 typically affords ~30 mg of protein per liter of expressed cells. The purified 13.4-kDa protein migrates with an apparent molecular mass of ~19 kDa in SDS-PAGE (not shown); this is likely a consequence of the acidic and hydrophilic nature of the protein (see Fig. 2a).³⁹ The UV/Vis absorbance spectrum of gpNu3 is notable in that it is devoid of absorbance at 280 nm (not shown), consistent with the lack of aromatic residues. This feature presents a challenge for accurate determination of protein concentration, which was addressed by amino acid compositional analysis as described in Materials and Methods.

The circular dichroism (CD) spectrum for gpNu3 indicates that the protein possesses significant α -helical structures as evidenced by the band at 222 nm. In addition, the presence of turns and disorder is evident by the intense band at 205 nm (Fig. 2b). The secondary-structure content in gpNu3 was quantified by spectral deconvolution using DichroWeb,⁴⁰ a CD spectral deconvolution server. Several algorithms and reference sets were utilized, and the best fit of the experimental data was achieved with CDSSTR⁴¹ and Contin⁴² (Supporting Information, Table S1). The analysis indicates that while λ gpNu3 possesses >50% helical structures, the protein also possesses significant disorder and turns. Decreasing the protein concentration 10-fold (64 μ M $\rightarrow$ 6.4 μ M) results in a slight decrease (10%) in the 222-nm CD band intensity (data not shown). These two concentrations span the apparent K_d for the monomer–dimer equilibrium (see below), and the data suggest that dimerization is associated with a modest increase in secondary structure. The 1D NMR spectrum of gpNu3 is also consistent with a protein that possesses helical character, little to no β -sheet structures, and segments of disorder (data not shown).

Limited proteolysis of gpNu3

The CD and NMR data suggest that gpNu3 possesses significant disorder, and we used limited proteolysis to probe for protease-sensitive regions of the protein. The data presented in Fig. 2d show that digestion of gpNu3 (9 μ M) with trypsin results in complete degradation of the full-length protein in about 5 min without generating any protease-resistant products. This suggests that gpNu3 possesses an “open” structure at this concentration. Increasing the protein concentration to 180 μ M does not significantly alter the digestion time course for the full-length protein; however, two stable fragments are observed under these conditions—an apparent 18-kDa fragment that is further degraded to an apparent 16-kDa product that remains stable for at least 60 min. We note that accurate calculation of the fragment sizes is complicated by the fact that full-length gpNu3 (13.4 kDa) migrates abnormally by SDS-PAGE (*vide supra*). This introduces uncertainty as to the molecular mass of the protease fragments based on their migration in the gel. Notwithstanding, the concentrations used in these experiments (9–180 μ M) span the apparent K_d for the monomer–dimer equilibrium (see below). This suggests that dimerization is associated with an increase in folded (and protease resistant) structure in the protein.

Thermal stability of gpNu3 secondary structures

Heating of gpNu3 results in a loss of the 222-nm CD band and a concomitant increase and shift of the 205-nm band to 200 nm (Fig. 2b). These changes reflect a loss of secondary structure and an increase in disordered content, respectively. Roughly 80% of the spectral features are recovered if the sample is cooled within 20 min from 75 °C (Fig. 2b). This indicates that unfolding is at least partially reversible; however, prolonged incubation (hours) at elevated temperature results in aggregation and precipitation of the protein (not shown).

We next determined the thermal stability of gpNu3, monitored by CD spectroscopy (222 nm band). The unfolding curve displays stable pre- and post-transition baselines, which represent folded and unfolded protein species, respectively. This behavior indicates that gpNu3 possesses a reasonably stable folded core; however, the transition is quite broad and suggests that protein unfolding is only weakly cooperative. A more complete thermodynamic analysis of the denaturation behavior of gpNu3 is presented in Supporting Information. In sum, the data indicate that gpNu3 not only contains α -helical structures and a folded core but also possesses significant disorder and flexibility.

Self-association behavior of gpNu3

Early studies suggested that gpNu3 is a monomer with a large Stokes radius, but the protein concentration was not specified.³⁹ Given that many scaffolding proteins self-associate,^{20,35,36,43} we pursued a rigorous examination of the solution properties of purified gpNu3. We first examined the self-association behavior of gpNu3 using SEC. The data show that the apparent Stokes radius of gpNu3 increases with increasing protein concentration (Fig. 3); this is a hallmark of self-association. Quantitative analysis of the SEC data presumes a spherical protein shape, which is clearly not the case for gpNu3 (*vide infra*). Thus, while these results demonstrate concentration-dependent self-association, an accurate determination of the association state is difficult and error prone due to the non-globular shape of the protein.

Sedimentation velocity analytical ultracentrifugation analysis of gpNu3

The solution self-association behavior of gpNu3 was next analyzed using sedimentation velocity analytical ultracentrifugation (SV-AUC) across a 10-fold concentration range (6–66 μ M). Initial analysis of the data was performed using the van Holde–Weischet (vHW) approach as implemented in UltraScan.⁴⁴ This method attempts to deconvolute diffusion from sedimentation by extrapolating transport processes to infinite time where sedimentation behavior dominates.⁴⁵ At 6 μ M gpNu3, the $s_{(20,w)}$ distribution indicates that a single, homogeneous 1.4-S species is present in solution (Fig. 4a)[†]. Increasing the gpNu3 concentration results in a gradual shift in the $s_{(20,w)}$ distribution from a 1.4-S species at 6 μ M to a 2.4-S species at 66 μ M (Fig. 4a). This is diagnostic for a reversible self-association interaction.⁴⁵ At an intermediate concentration of 37 μ M, the observed $s_{(20,w)}$ distribution lies between these two values. This reflects a weight average of the species in solution. The concentration-dependent $s_{(20,w)}$ distribution suggests a rapid equilibrium relative to the timescale of the experiment (as opposed to observing two distinct species) and is consistent with the peak shifting observed in SEC (Fig. 3). Finally, we note the presence of negative curvature in the upper boundary fractions of the vHW plots. This behavior is diagnostic for either (i) nonideal behavior or (ii) loss of material during the centrifugation run.⁴⁵ The extrapolation plots do not exhibit unusual concentration-dependent convergence (not shown), which disfavors the former explanation. Further, a small amount of material (10%) is typically lost during the experiment. Thus, the observed negative curvature observed in the upper boundaries of the 37 and 66 μ M samples likely represents the loss of protein that has aggregated during the 8-h analysis.⁴⁵

The vHW analysis clearly demonstrates that gpNu3 undergoes a reversible, concentration-dependent self-association interaction; however, it is not possible to extract rate or equilibrium information from this analysis.⁴⁵ We therefore analyzed the data using the $c(s)$ model as implemented in SEDFIT.⁴⁶ This analysis fits a frictional ratio to correlate diffusion

[†]The maximal rotor speed allowed by the Eponcharcoal centerpieces (50,000 rpm) was utilized in these studies; this was, however, slow relative to the small size of the gpNu3 monomer. Thus, the “tail” present at lower boundary fractions is likely due to diffusion affecting the vHW analysis.⁴⁵

and mass in an effort to account for diffusion during the SV runs. The $c(s)$ distribution shows that a single 1.5-S species predominates at a protein concentration of 6 μM (Fig. 4b), consistent with the vHW analysis. A frictional ratio (f/f_0) of 1.4 may be directly determined under these conditions. This value indicates that the protein possesses an asymmetric shape and predicts an axial ratio of 5.5 for a prolate ellipsoid.⁴⁷ Analysis of the data affords a molecular mass of 15.3 kDa (1σ confidence=13.5–17.4 kDa). This indicates that the protein is predominantly a monomer at this concentration (molecular mass from gene sequence is 13.4 kDa)[§].

Increasing the gpNu3 concentration to 37 μM affords a second (2.5 S) species (Fig. 4b). Further increasing the gpNu3 concentration to 66 μM results in a slight increase in the calculated $s_{(20,w)}$ values for the two species (1.7 and 2.8 S) and shifts the equilibrium towards the larger species. Analysis of the data yields a calculated molecular mass of 32.4 kDa (1σ confidence=31.1–34.9 kDa) for the larger species. This is double the molecular mass for the monomer obtained above, thus identifying it as a dimer of gpNu3^{||}. We note that while SEDFIT affords discrete peaks in the $c(s)$ distribution, the position of the peaks shift to higher s values with increasing protein concentration. This is consistent with the vHW analysis, which shows a gradual concentration-dependent shift in the s distribution (see Fig. 4a). In sum, the data indicate that the association kinetics play a role on the timescale of the experiment. A global analysis of the $c(s)$ distributions at all three concentrations was performed using SEDPHAT,⁴⁸ which affords a gpNu3 dimerization constant ($K_{d,app}$) of 50 μM (1σ confidence=10–150 μM). Increased ionic strength (up to 500 mM NaCl) only modestly affected the $K_{d,app}$ (~10% decrease; data not shown).

GpNu3 chaperones the gpE major capsid protein into VLPs

We next examined the biological activity of purified gpNu3 using a defined *in vitro* capsid assembly system. The purified gpE major capsid protein (15 μM) is soluble in the presence of 0.5 M guanidine hydrochloride (GDN) but forms insoluble aggregates upon further dialysis of the GDN from solution (data not shown). In contrast, capsid protein remains soluble when gpNu3 is included in the mixture. Under these conditions, the bulk of the soluble gpE elutes near the void volume of the column (Peak 1, Fig. 5a). This indicates that the capsid protein has been incorporated into soluble, higher-order complexes. Heating gpNu3 to 80 °C for 5 min does not significantly affect its ability to catalyze VLP assembly (data not shown); this is consistent with reversible thermal denaturation of the protein (see Fig. 2b).

Increasing the gpNu3:gpE ratio from 0 to 0.5:1 results in an increase in the amount of soluble protein eluting in Peak 1 (Fig. 5a, inset). Further increasing gpNu3 results in gpE precipitation from the mixture (not shown) and a decrease in the quantity of soluble complexes (Fig. 5a). The salt dependence of the assembly reaction was investigated, and Fig. 5c shows that optimal particle assembly is observed at 150 mM NaCl. Similar salt effects have been observed for the *in vitro* assembly of VLPs in other bacteriophage systems.^{20,21,23} Examination of material eluting in Peak 1 by SDS-PAGE (Fig. 5b) clearly

[§]The observed $c(s)$ distribution is broad, which may be attributed to heterogeneity in the sample, diffusion, and/or noise in the raw data. Since the vHW analysis indicates that the gpNu3 sample is homogeneous (Fig. 4a) and the noise in the data is not excessive (not shown), the most parsimonious explanation is significant diffusion during the run; this interpretation is consistent with the vHW analysis. We note that this will affect accurate determination of the frictional ratio and thus the calculated molecular mass of the species,⁴⁶ as is observed here.

^{||}The calculated molecular mass for the 2.8-S species could in fact accommodate either a dimer (26.8kDa) or a trimer (40.2kDa) of gpNu3; however, accurate molecular mass determination by SV-AUC is highly dependent on accurate quantitation of the frictional ratio,⁴⁶ which is error-prone as described in the previous footnote. That said, the calculated molecular mass of the 2.8-S species is essentially double that of the 1.5-S species, and we interpret the data to indicate that the 2.8-S species represents a dimer of gpNu3. Consistently, analysis of the data using a monomer–dimer model afforded a significantly better fit than a monomer–trimer model (not shown).

demonstrates that gpE elutes in this fraction when mixed with gpNu3. In contrast, the capsid protein elutes as a monomer in the absence of the scaffolding protein (Peak 2). Likewise, the scaffolding protein elutes with VLPs in the presence of gpE but not in its absence. We note that the ratio of gpNu3:gpE eluting in Peak 1 is quite low under all conditions (Fig. 5b). Previous studies have demonstrated that full-length gpNu3 can exit from a wild-type procapsid *in vitro*¹⁹ and *in vivo*.³¹ The present data suggest that gpNu3 can similarly exit the VLP interior once the shell has been assembled.

The material eluting in Peak 1 of the chromatogram was further examined by electron microscopy. The micrographs clearly show that gpE is assembled into VLPs in the presence of gpNu3 (Fig. 6). The concentration of gpE included in the assembly mixtures was held constant, and the amount of material eluting in Peak 1 is thus an indication of VLP formation efficiency but not fidelity. Electron microscopy provides an indication of the quality of gpE assembly into the structures. For instance, in the absence of gpNu3, the minor amount of gpE eluting in Peak 1 is primarily assembled into smaller, aberrant complexes (Fig. 6). Addition of gpNu3 to the mixture promotes the assembly of gpE into higher-order structures with maximal efficiency achieved at a gpNu3:gpE ratio of ~0.5:1 (high-yield conditions; Fig. 5a); however, assembly of uniform VLP structures occurs at a lower ratio of only 0.3:1 (Table 1). This yields procapsid-like particles with a radius of 46 ± 5.2 nm (high-fidelity conditions; Fig. 6). Under these conditions, the *in vitro* assembled VLPs retain the $T=7$ symmetry of *bona fide* λ procapsids.^{16,49} At higher ratios, a collection of aberrant extended structures that eventually precipitate from the reaction mixture is observed. We note that gpNu3 is predominantly a monomer under conditions that support high-fidelity VLP assembly (gpNu3:gpE ratio, 1:3 $\rightarrow$ ~4.5 μ M gpNu3; dimerization $K_{d,app}$ ~50 μ M).

Discussion

The assembly of complex dsDNA viruses includes a genome-packaging step where viral DNA is translocated into a procapsid structure by a terminase motor complex.^{4,6,7} Procapsids self-assemble by co-polymerization of a major capsid protein and an internal scaffolding protein. The scaffold protein serves as catalyst and chaperone to direct capsid shell formation and closure into an icosahedral structure.^{11–13} In the absence of scaffolding protein, the major capsid proteins assemble into aberrant structures into which the genome cannot be packaged. This is a lethal event, and scaffolding proteins are thus essential to the development of dsDNA viruses. Characterization of their structural, biophysical, and functional properties is necessary for an understanding of virus assembly in the cell. To complement and extend our present understanding of these essential viral proteins, we describe in this study a comprehensive biophysical and functional characterization of the λ gpNu3 scaffolding protein. We note that λ packages a “unit length” genome into the procapsid shell. This is similar to the herpesviruses but contrasts to the “headful” packaging mechanism observed in phages P22 and SPP1 and the monomeric genome-packaging mechanism observed in ϕ 29.⁶ Thus, the results reported here define procapsid assembly in a related but distinct virus class.

Structure and self-association behavior of the gpNu3 scaffolding protein

Primary sequence analysis, CD and NMR spectroscopy, and limited proteolysis data indicate that the λ gpNu3 protein possesses a loosely folded helical core and is partially disordered. The core is moderately stable to thermal denaturation but unfolds with limited cooperativity. Thus, gpNu3 possesses features consistent with an intrinsically disordered protein — inherent flexibility and structural disorder.³⁸ We note that the λ scaffolding protein appears to be more disordered than the other characterized scaffolding proteins that typically show higher helical content.^{20,21,36} GpNu3 also possesses an elongated shape with an axial ratio of 5.5. While this could reflect structural disorder as suggested for the phage P22 and SPP1

proteins (axial ratios ~10 and ~20, respectively),^{35,36} this value is virtually identical with that observed in the crystal structure of the $\phi 29$ scaffolding protein.⁵⁰ This suggests that portions of gpNu3 are composed of folded domains, which is supported by limited proteolysis data.

A helix–turn–helix structural motif has been identified in the scaffolding proteins of P22⁵¹ and $\phi 29$.⁵⁰ This “capsid binding” motif has been proposed to be a common structural feature of all the scaffold proteins.^{11–13} We searched for evidence of this putative motif in gpNu3; unfortunately, neither primary sequence analysis (BLAST®), secondary-structure prediction (SABLE®, COILS®), nor protein fold recognition analysis (Phyre®, I-TASSER®) identified any functional domains or significant homology to any known scaffolding protein. This is further complicated by the fact that the helix–turn–helix motif in the P22 and $\phi 29$ scaffolds are found in opposite ends of the protein. Although sequence analysis does not reveal significant homology between the scaffold proteins, we presume that a similar helix–turn–helix motif is ensconced within the gpNu3 dimer.

Despite significant structural disorder, gpNu3 exists in a monomer–dimer equilibrium with a $K_{d,app}$ ~50 μ M. Interestingly, dimerization of gpNu3 is associated with an increase in the folded structure of the protein. The $K_{d,app}$ measured here is remarkably similar to the monomer–dimer equilibrium constant observed in the P22 system (43 μ M).³⁵ In contrast, the phage SPP1 scaffolding protein is a tetramer at these concentrations.³⁶ SEC data demonstrate that higher-order associations occur at elevated gpNu3 concentrations, which has also been observed with the P22 scaffolding protein.³⁵ We did not characterize this in detail because the higher-order assembly occurs in a non-biologically relevant concentration range[¶]. In sum, the gpNu3 scaffolding protein shares the essential structural and hydrodynamic features of other characterized scaffolding proteins despite the lack of sequence homology.

Biological activity of gpNu3

Early studies demonstrated that gpNu3 can complement crude extracts of a λ *Nu3*[−] lysogen.³⁹ Here, we show that purified gpNu3 efficiently chaperones the gpE major capsid protein into VLPs *in vitro*. The isolated gpE protein shows a strong tendency to self-assemble into amorphous, insoluble aggregates; however, gpE remains soluble and assembles into VLPs in the presence of gpNu3. The stoichiometry of the reaction can be estimated from the yield and morphology of the particles as a function of the molar ratio of gpNu3:gpE. A ratio of 0.3:1 affords the greatest fraction of VLPs that possess morphology similar to *bona fide* procapsid structures. Increasing gpNu3 to a ratio of 0.5:1 affords the maximal yield of soluble particles, but they are irregular and many possess an extended, tubelike morphology. Further increasing the concentration of gpNu3 results in precipitation of capsid protein from solution. In sum, our data indicate that (i) the gpNu3 scaffolding protein functions to control the yield of capsid assembly with gpE, (ii) it functions to ensure that the assembly process proceeds along a productive pathway leading to a closed icosahedral shell of defined shape and size, and (iii) there is a delicate balance between the two processes.

The gpNu3 concentration required for optimal VLP assembly is on the order of that found in the infected *Escherichia coli* cell[¶].^{52,53} This indicates that while gpNu3 is predominantly a monomer *in vivo*, a small population of dimer is always present ($K_{d,app}$ = 50 μ M). In fact, scaffold protein dimers have been proposed to nucleate procapsid assembly in phages P22,³⁵

[¶]The concentration of gpNu3 in an infected cell (10–60 μ M) can be estimated from the presumed concentration of procapsids (150–300 nM⁵³) and assuming that 70–200 scaffolding proteins are found in the nascent shell.^{30,31}

SPP1,³⁶ and $\phi 29$ ⁵⁰ and in the herpesviruses.^{25,54,55} Our data are consistent with a dynamic equilibrium that ensures a relatively low but constant supply of the gpNu3 dimer. Further, this dynamic equilibrium would ensure that an ample population of monomer is always present. It is generally accepted that an appropriate distribution of dimer and monomer is required to balance nucleation and shell growth, respectively.^{9,10,35} Indeed, an excess of scaffolding protein affords partially formed shells in many viral systems including phage P22⁵⁶ and phage T7.²³ It has been proposed that excess scaffolding protein “overdrives” a nucleation event, ostensibly mediated by the scaffolding protein dimer. The result is a rapid depletion of capsid protein monomers from solution such that capsid shell polymerization cannot be completed (a kinetic trap). This is phenotypically distinct from the λ system where too much scaffold protein yields large and irregular structures that precipitate from solution. We suggest that aberrant assembly in λ results from self-association of gpNu3 beyond the dimer state during co-polymerization with capsid protein; this could have several consequences. First, higher-order gpNu3 oligomers could present an elongated surface that promotes aberrant polymerization of capsid protein. Scaffold-mediated “conformational switching” of the incoming capsid protein to an assembly-competent state has been proposed in other systems.^{12,57,58} GpNu3 oligomers could affect co-polymerization with the incoming capsid proteins such that shell closure is precluded. Second, gpNu3 oligomers could have increased affinity for capsid protein monomers as they are incorporated into the nascent shell. This has been postulated in the P22 system where too much scaffold protein affords partial shells.⁵⁶ In the case of λ , however, increased capsid affinity could result in overdriving the elongation phase of shell growth. Multiple, diverse, and weak interactions are at the heart of scaffold–capsid polymerization into an icosahedral capsid, as discussed above. Fidelity requires that appropriate scaffold–scaffold, scaffold–capsid, and capsid–capsid interactions be maintained during the course of shell assembly. An inappropriately incorporated capsid protein must therefore be released or provided sufficient time for a putative conformational change prior to the addition of additional monomers. Otherwise, subsequent addition of capsid proteins in a non-physiological conformation would result in uncontrolled assembly of aberrant structures. These defective binding events would typically be “corrected” by the readily reversible nature of capsid assembly (e.g., weak protein interactions). If the scaffold lattice binds too tightly, release of inappropriate conformational intermediates cannot occur or may occur too slowly. This would abrogate a kinetic fidelity step and push capsid assembly through flawed assembly pathway.

General principles governing capsid assembly

Viral capsids are assembled from one or only a few capsid proteins.^{59,60} Even in those cases where the entire capsid shell is composed of a single protein, multiple “quasi-equivalent” conformational states must be adopted to assemble a closed shell composed of hexameric faces and pentameric vertices.^{11,49,59} This requires that the scaffolding proteins bind to conformationally nonequivalent capsid proteins during coupled co-polymerization. Furthermore, the scaffolding proteins must undergo self-assembly interactions during shell polymerization. All of these interactions must be specific yet transient, sufficiently strong to catalyze shell assembly, yet weak enough to accommodate scaffolding protein release from the assembled procapsid. Further, this departure must occur without disruption of the established interactions between the capsid proteins assembled into the shell. “Intrinsic disorder,” as observed in gpNu3, may reflect a structural plasticity required to correctly assemble an icosahedral shell and subsequently release the scaffolding protein from the procapsid interior. Indeed, coupled folding of disordered protein domains upon binding to their biological targets can play an important role in the assembly of macromolecular arrays.³⁸ This provides a mechanism for a protein to adopt alternate conformations required for specific interactions with multiple partners.^{38,61,62}

The combination of a weak monomer–dimer equilibrium combined with intrinsic structural disorder may be key to the function of the scaffold proteins in capsid assembly. Despite their lack of primary sequence similarity and distinct differences in size (100 to 300 residues), the scaffold proteins share fundamental features including elongated shape, flexibility, disorder, and weak self- and capsid-binding interactions. This likely reflects the fact that the essential features of procapsid assembly are conserved among all of the complex dsDNA viruses, both prokaryotic and eukaryotic. The physical features of gpNu3 directly demonstrated here—flexibility, partial disorder, and weak self-association—are ideally suited to its biological role in capsid assembly.

Materials and Methods

Materials

Tryptone, yeast extract, and agar were purchased from DIFCO. All chromatography media were purchased from GE Healthcare. Goat anti-rabbit fluorescein conjugate antibody was purchased from Upstate Biotechnology (now part of Millipore) and gpNu3 polyclonal antibody was a generous gift of Dr. Michael Feiss (Department of Microbiology, University of Iowa). Western blot analysis was performed as previously described.¹⁹ All other materials were of the highest quality available.

Bacterial cultures were grown in shaker flasks utilizing an Innova 4430 Incubator Shaker. All protein purifications utilized the Amersham Biosciences ÄKTApurifier core 10 system from GE Healthcare. UV/Vis absorbance spectra were recorded on a Hewlett-Packard HP8452A spectrophotometer. Video images were captured using an EpiChem³ darkroom system with a Hamamatsu camera (UVP Bioimaging Systems). Electron micrographs were obtained on an FEI Morgagni 268 100-kV electron microscope as previously described.¹⁹

Expression and purification of gpE

The bacteriophage λ gpE protein was purified as described previously,¹⁹ with modification. Briefly, the induced cells were harvested by centrifugation, and the cell pellet was resuspended in 50 mL of ice-cold 20 mM Tris buffer, pH 8, containing 1 mM ethylenediaminetetraacetic acid and 20% sucrose; unless otherwise indicated, all subsequent steps were performed with ice-cold buffers at 0 to 4 °C. The cells were gently mixed for 10 min and harvested by centrifugation (9000g for 30 min). The cell pellet was resuspended in 50 mL of ice-cold water and gently stirred for 10 min, and the cells were again harvested by centrifugation (12,000g for 40 min). This hypertonic–hypotonic treatment removes outer cell membrane proteins that would otherwise contaminate the preparation.⁶³ The spheroplasts were resuspended in 20 mL of Buffer A (50 mM Tris buffer, pH 8, 5 M GDN, 100 mM NaCl, and 10 mM MgCl₂) containing 10 mM imidazole; the cells were lysed by sonication (10×10-s bursts) and gently stirred overnight at 4 °C. Insoluble proteins were removed by centrifugation (12,000g for 30 min), and the supernatant was applied to a GE HisTrap FF Ni-NTA column (5 mL) that had been equilibrated with Buffer A. The column was washed with Buffer A, and the bound material was eluted with a linear gradient to 500 mM imidazole. The column fractions were examined for the presence of gpE by SDS-PAGE, and appropriate fractions were pooled; gpE was eluted with 50 mM imidazole. The protein concentration in the pooled sample was reduced to 20 μ M or less by dilution with Buffer A to minimize aggregation, and the GDN concentration was then reduced to 0.5 M through a four-step dialysis against Buffer B [50 mM Tris buffer, pH 8, containing 300 mM NaCl and 7 mM beta-mercaptoethanol (β -ME)] and decreasing concentrations of GDN (3, 2, 1, and 0.5 M); the purified protein was stored at 4 °C in Buffer B containing 0.5 M GDN. The purity of the preparation was >95% as determined by SDS-PAGE, and the concentration of the protein was determined spectrally using an extinction coefficient (ϵ_{280})

of $36,330 \text{ M}^{-1} \text{ cm}^{-1}$ (in Buffer B containing 0.5 M GDN) determined using the Edelhoch method.⁶⁴

Expression and purification of gpNu3

E. coli BL21(DE3) cells were transfected with the plasmid pT7Init-gpC-S166A, which expresses the λ viral proteins gpB, gpC-S166A (defective protease), and gpNu3,¹⁹ and cultured overnight at 37 °C on LB plates containing 50 $\mu\text{g mL}^{-1}$ of ampicillin. A single bacterial colony was used to inoculate 25 mL of 2 $\times$ YT media containing 50 $\mu\text{g mL}^{-1}$ of ampicillin, and the culture was maintained at 37 °C overnight. This culture was used to inoculate 1 L of 2 $\times$ YT media, pH 7.2, containing 25 mM sodium phosphate, 5 mM glucose, and 50 $\mu\text{g mL}^{-1}$ ampicillin, and the culture was shaken at 200 rpm at 37 °C until an optical density at 600 nm of 0.5 was obtained. Protein expression was then induced with 1 mM IPTG, the culture was shaken at 37 °C for 2 h, and the cells were harvested by centrifugation. The cell pellet was resuspended in 25 mL of ice-cold Buffer C (20 mM Tris buffer, pH 8, containing 5 mM MgCl_2 and 5 mM β -ME); unless otherwise indicated, all subsequent steps were performed with ice-cold buffers at 0 to 4 °C. The cells were lysed by sonication (10 $\times$ 10-s bursts), lysozyme was added to a concentration of 80 $\mu\text{g mL}^{-1}$, and 500 μL of ethylenediaminetetraacetic-acid-free 100 $\times$ Thermo Protease inhibitor (Thermo Scientific) was added to the mixture, which was maintained at 4 °C for 1 h. Insoluble material was removed by centrifugation (9000g for 20 min), and solid ammonium sulfate was added to the lysis supernatant to 20% saturation. The sample was gently stirred on ice for 45 min, and insoluble protein was removed by centrifugation (9000g for 25 min).

Ammonium sulfate was then added to the supernatant to 70% saturation, the sample was stirred for 45 min, and the insoluble protein was harvested by centrifugation (9000g for 25 min). The 70% ammonium sulfate pellet was resuspended in 25 mL of Buffer C, dialyzed against 1 L of Buffer C, and loaded onto a 50-mL diethylaminoethyl-Sepharose column equilibrated with the same buffer. The column was washed with Buffer C containing 100 mM NaCl, and the bound material was eluted with a linear gradient to 500 mM NaCl. Fractions were examined for the presence of gpNu3 by SDS-PAGE, which eluted as a broad peak between 100 and 250 mM NaCl. The gpNu3-containing fractions were pooled, dialyzed against Buffer C, and loaded onto a 6-mL GE Resource Q column equilibrated with the same buffer. Bound proteins were eluted with a linear gradient to 350 mM NaCl, and column fractions were examined for the presence of gpNu3 by SDS-PAGE; gpNu3 was eluted with 150 mM NaCl. Fractions containing gpNu3 were pooled, dialyzed against 20 mM Tris buffer, pH 8, containing 100 mM NaCl and 5 mM MgCl_2 , and stored at 4 °C. The purity of the preparation was >95% pure as determined by SDS-PAGE.

Determination of gpNu3 protein concentration

gpNu3 is devoid of aromatic residues which precludes the use of the Edelhoch method to determine protein concentration. Therefore, the purified preparation was submitted for amino acid analysis, which was performed on a fee-for-service basis at the Purdue University Proteomics Facility (Bindley Bioscience Center). The concentration of gpNu3 in a stock solution was determined in two separate analyses, and the data were averaged to afford a gpNu3 concentration of $8.3 \pm 0.15 \text{ mg mL}^{-1}$. This stock solution was used to derive an extinction coefficient (ϵ_{230}) of $20,140 \text{ M}^{-1} \text{ cm}^{-1}$ in 20 mM phosphate, 100 mM NaCl, and 5 mM MgCl_2 . This extinction coefficient was subsequently used to determine gpNu3 protein concentration.

CD spectroscopy

CD spectra were collected on an Aviv 62 A-DS spectrophotometer (Aviv Biomedical, Inc., Lakewood, NJ) in a jacketed cell holder. Unless otherwise specified, spectra were acquired

at 20 °C in a 1-mm strain-free quartz cuvette using a protein concentration of 64 μ M in 20 mM sodium phosphate buffer, pH 7.5, containing 100 mM NaCl and 5 mM MgCl₂. Data were collected at 1-nm intervals using a bandwidth of 1 nm and a dwell time of 3 s. The raw data were converted to molar ellipticity using,

$$\Theta = \Theta_{\text{obs}} \left(\frac{\text{MRW}}{10bc} \right) \quad (1)$$

where Θ is the molar ellipticity (millidegrees per square centimeter per decimole), Θ_{obs} is the ellipticity recorded by the instrument (millidegrees), MRW is the mean residue weight (formula weight divided by the total number of residues in the protein), b is the cell path length in centimeters, and c is the protein concentration in milligrams per milliliter.

Thermal denaturation experiments

Thermally induced protein denaturation was monitored by far-UV CD spectroscopy. Protein at a concentration of 64 μ M in 20 mM sodium phosphate buffer, pH 7.5, containing 100 mM NaCl and 5 mM MgCl₂, was placed in a 1-mm strain-free quartz cuvette, and data were collected at the temperature indicated in each individual experiment. Full spectra were recorded from 260 to 190 nm at intervals of 1 nm with a dwell time of 3 s; a buffer blank was subtracted from all data sets. For thermal denaturation studies, the temperature was increased in 1 °C increments at a rate of 2 °C per minute, the sample temperature was allowed to equilibrate for 60 s (unless otherwise noted), and the ellipticity at 222 nm was then time averaged for 12 s. A further thermodynamic analysis is described in Supporting Information.

Limited proteolysis of gpNu3

The protease reaction mixtures contained gpNu3 at either 9 or 180 μ M in 20 mM Tris, pH 8, 100 mM NaCl, and 5 mM MgCl₂ buffer. Trypsin (1:200 mass ratio) was added, and the reaction was allowed to proceed at room temperature. Aliquots were removed at the indicated times, and the reaction was quenched with addition of reducing SDS-PAGE loading buffer and boiling for 5 min. The quenched reaction mixtures were analyzed by 15% SDS-PAGE stained with Coomassie blue.

Size-exclusion chromatography

Analytical SEC utilized a GE Superose 6 10/300 GL column (24 mL) equilibrated with 20 mM Tris buffer, pH 8, containing 100 mM NaCl and 5 mM MgCl₂ at a flow rate of 0.5 mL min⁻¹. The column was calibrated using the GE Gel Filtration Calibration Kit [ribonuclease A (13.7 kDa, 16.4 Å), ovalbumin (43.0 kDa, 30.5 Å), conalbumin (75.0 kDa, 40.4 Å), alcohol dehydrogenase (150 kDa, 45 Å), and apoferritin (440 kDa, 61 Å) (GE Healthcare SEC handbook)].⁶⁵ Void volume (V_0) and total column volume (V_T) were determined using blue dextran and acetone, respectively. The elution volume (V_e) for each protein standard was determined from the average of at least three separate injections and used to calculate the partition coefficient,

$$K_{\text{av}} = \left(\frac{V_e - V_0}{V_T - V_0} \right) \quad (2)$$

Molecular weight and Stokes radius standard curves were constructed by plotting K_{av} versus $\log(MW)$ and Stokes radius versus $[-\log(K_{av})]^{1/2}$, respectively.⁶⁶

Purified gpNu3 at the indicated concentration was injected onto the calibrated SEC column, and elution of the protein was monitored by absorbance at 230 nm. The K_{av} at each concentration was determined from the average of at least three separate injections using Eq. (2). The apparent molecular weight and Stokes radius for each concentration of gpNu3 were determined from the standard curves as described above.

AUC studies

Unless otherwise specified, protein samples (400 μ L) at the indicated concentration in 20 mM Tris–HCl buffer, pH 8, containing 100 mM NaCl and 5 mM MgCl₂ were loaded into the sample chamber of a 12-mm Epon charcoal two-sector centerpiece. The samples were prepared either the night before or immediately prior to centrifugation, and the concentration was determined by UV spectroscopy at 230 nm; dialysate buffer was used for the reference chamber of the cell. SV experiments were performed at 20 °C and at a rotor speed of 50,000 rpm in a Beckman XL-A analytical ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA). Absorbance data were collected at 230 nm, using a spacing of 0.001 cm, with four averages in the continuous scan mode; scans were collected every 15 min. The raw data were analyzed using both the UltraScan comprehensive data analysis suite⁴⁴ and the SEDFIT/SEDPHAT data analysis packages.^{46,48}

Assembly of VLPs *in vitro*

Purified gpE major capsid protein (15 μ M) was mixed with varying concentrations of purified gpNu3 in a 2-mL reaction volume still containing 0.5 M GDN to minimize aggregation, and the mixtures were dialyzed overnight at 4 °C against 50 mM Tris buffer, pH 8, containing 300 mM NaCl, 15 mM MgCl₂, and 7 mM β -ME. The samples were then concentrated to 500 μ L and loaded onto a Superose 6 HR10/300 GL column equilibrated with 50 mM Tris buffer, pH 8, containing 150 mM NaCl, 15 mM MgCl₂, and 7 mM β -ME; the column was developed with the same buffer at a flow rate of 0.5 mL min^{−1}. Load samples and chromatographic peak fractions were analyzed by SDS-PAGE, Western blot, and electron microscopy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Dr. Jenny Chang for help with some of the VLP assembly data and for helpful discussions of this work. We would also like to thank Dr. David Baker for use of the CD spectrophotometer and Dr. Tamir Gonen for assistance and guidance with the use of the electron microscope. This work was supported by National Science Foundation grant MCB-0648617 (C.E.C.) and National Institutes of Health grant F32GM-905652 (B.T.A.).

Abbreviations used

β -ME	beta-mercaptoethanol
dsDNA	double-stranded DNA
GDN	guanidine hydrochloride
SEC	size-exclusion chromatography

SV	sedimentation velocity
AUC	analytical ultracentrifugation
VLP	virus-like particle
vHW	van Holde–Weischet

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Fig. 1.
Model for the assembly of a λ procapsid. Details are provided in the text.

Fig. 2.

The gpNu3 protein possesses α-helix and random-coil secondary structures. (a) Secondary-structure prediction of gpNu3 using the SABLE protein structure prediction server (<http://sable.cchmc.org>). (b) Far-UV CD spectra of purified gpNu3 (64 μM) recorded at 4 °C (●) shows bands at 205 and 222 nm, consistent with random-coil and α-helix structures, respectively. Spectral deconvolution prediction results using CDSSTR are shown as a continuous line (reference set 4; Supplemental, Table S1). CD spectra of gpNu3 heated to 75 °C for 20 min (▲) and then cooled to 4 °C (◆) indicates >80% recovery of helical structures upon refolding. (c) Thermally induced denaturation of gpNu3 (64 μM) was monitored by CD spectroscopy (222 nm). The melting curves have a cooperative transition,

indicative of a folded structure. The data were analyzed as described in Supporting Information (continuous line), which afforded a $T_m = 40.6 \pm 0.3$ °C and $m^T = 5.0 \pm 0.2$. Longer equilibration times (3 min *versus* 1 min) had no effect on results, confirming equilibrium.

(d) Limited proteolysis of gpNu3 with trypsin. GpNu3 at a concentration of 9 and 180 μ M, as indicated, was digested with trypsin as described in Materials and Methods. The migration of full-length gpNu3 is indicated with an asterisk. The two stable fragments observed at elevated gpNu3 concentrations are indicated with arrows.

Fig. 3.

SEC analysis suggests that gpNu3 possesses an extended conformation and self-associates in a concentration-dependent manner. Stokes radius as a function of gpNu3 concentration was determined by SEC as described in Materials and Methods. Inset shows the elution profile of gpNu3 as a function of protein concentration (4.5 μM, red dotted line; 8 μM, blue broken line; 50 μM, magenta broken line; 230 μM, black continuous line).

Fig. 4.

SV analysis demonstrates that gpNu3 associates in a monomer–dimer equilibrium. (a) vHW analysis was performed and the data demonstrate that gpNu3 is a homogeneous 1.4 S species at 6 μM ($\blacktriangle$) and self-associates with increasing concentration ($\blacksquare$, 37 μM ; $\bullet$, 66 μM). The gradual shift in s value is consistent with a rapid equilibrium relative to the timescale of the experiment. (b) A $c(s)$ analysis of the same data was performed using Tikhonov–Phillips regularization and a 68% confidence level to calculate the distributions. The two observed species represent a 1.4-S monomer and a >2.8 -S dimer. Increasing the concentration of gpNu3 results in an increase in proportion of the larger species indicative of a protein that self-associates. A global fit of the three data sets yields a $K_{d,app} = 50 \mu\text{M}$ (1σ confidence=10–150 μM).

Fig. 5.

gpNu3 promotes assembly of the λ major capsid protein (gpE) into large complexes. (a) SEC chromatograms of reaction mixtures containing gpE (15 μ M) and increasing concentrations of gpNu3 (black continuous line, none; maroon continuous line, 1.5 μ M; blue continuous line, 4.5 μ M; green continuous line, 7.5 μ M; maroon broken line, 15 μ M; blue broken line, 30 μ M). Peak 1 (7 mL) elutes in the void volume of the column while Peak 2 (17 mL) elutes at a position consistent with monomeric gpE (38 kDa). Note that gpNu3 does not absorb at 280 nm and is not observed in the chromatograms. Inset: Peak height of Peak 1 as a function of the gpNu3:gpE ratio in the reaction mixture. (b) Load samples and peak fractions from various gpNu3:gpE ratios in (a) were analyzed by SDS-PAGE stained with Coomassie blue. (c) Salt inhibits VLP assembly. The assembly reaction was performed using a gpNu3:gpE ratio of 0.3:1 with additional salt as indicated.

Fig. 6. gpNu3 promotes gpE assembly into VLPs. Material eluting in Peak 1 of the chromatograms displayed in Fig. 5a was collected and examined by electron microscopy (14,000 $\times$). The ratio gpNu3:gpE is indicated in each panel. Although a maximal yield of particles is obtained with a ratio of 0.5:1 (Fig. 5a), optimal fidelity of VLP assembly is observed at a ratio of 0.3:1; elevated ratios afford grossly misshapen particles. The inset in the top left panel shows a micrograph of wild-type λ procapsids at the same scale.

Table 1Fidelity of VLP assembly *in vitro*

gpNu3:gpE ratio	Particles counted	Normal VLPs ^a (%)	Damaged VLPs ^b (%)	Aberrant VLPs ^c (%)
0:1	12	8	ND ^d	92
0.1:1	100	69	20	11
0.3:1	312	72	17	11
0.5:1	288	48	35	17
1:1	103	22	58	20
2:1	59	44	31	25

^a Normal VLPs were between 45 and 55 nm in diameter and possessed a morphology similar to that of intact λ procapsids (see Fig. 6).

^b Damaged VLPs were flattened or slightly fragmented (presumably during staining) but had no other obvious structural defects.

^c Aberrant VLPs were incompletely formed or elongated structures.

^d ND, none detected.