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Electrostatic interactions govern both nucleation and elongation during phage P22 procapsid assembly

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Abstract

Icosahedral capsid assembly is an example of a reaction controlled solely by the interactions of the proteins involved. Bacteriophage P22 procapsids can be assembled in vitro by mixing coat and scaffolding proteins in a nucleation-limited reaction, where scaffolding protein directs the proper assembly of coat protein. Here, we investigated the effect of the buffer composition on the interactions necessary for capsid assembly. Different concentrations of various salts, chosen to follow the electroselectivity series for anions, were added to the assembly reaction. The concentration and type of salt was found to be crucial for proper nucleation of procapsids. Nucleation in low salt concentrations readily occurred but led to bowl-like partial procapsids, as visualized by negative stain electron microscopy. The edge of the partial capsids remained assembly-competent since coat protein addition triggered procapsid completion. The addition of salt to the partial capsids also caused procapsid completion. In addition, each salt affected both assembly rates and the extent of procapsid formation. We hypothesize that low salt conditions increase the coat protein:scaffolding protein affinity, causing excessive nuclei to form, which decreases coat protein levels leading to incomplete assembly.

Keywords: Virus; Ionic; Subunit interactions; Light scattering; Hofmeister

Introduction

Bacteriophage and viruses are complex macromolecular structures that are capable of self-assembly. For bacteriophage P22, a dsDNA phage of *Salmonella enterica* serovar Typhimurium, much is understood about the assembly process. In vivo assembly involves the co-polymerization of 420 molecules of coat protein (gp5) and 150–300 molecules of scaffolding protein (gp8) (Casjens, 1979; Casjens and King, 1974; Eppler et al., 1991; Parent et al., 2004; Prasad et al., 1993; Thuman-Commike et al., 1996). In addition to coat protein and scaffolding protein, a portal complex composed of a ring of 12 portal proteins (gp1) is also incorporated at one of the penton positions during assembly into a non-infectious, precursor structure called a procapsid (Bazinet et al., 1988; Moore and Prevelige, 2002). Three other proteins, called injection proteins (sometimes referred to as pilot proteins) gp7, gp16, and gp20, have 10–20 copies each incorporated into the procapsid during assembly (Bryant and King, 1984; Thomas and Prevelige, 1991; Umlauf and Dreiseikelmann, 1992). After the procapsid is assembled, double-stranded DNA is packaged via the portal vertex, and concomitantly scaffolding protein exits, likely through holes in the coat protein lattice (Bazinet and King, 1985; Prasad et al., 1993).

Though P22 procapsid assembly has been characterized in general, control of the nucleation and elongation reactions is not completely understood. Purified coat and scaffolding proteins are necessary and sufficient for assembly of procapsid-like structures in vitro (Fuller and King, 1982; Prevelige et al., 1988, 1993). The resulting macromolecular structures have the same dimensions and overall gross morphology as in vivo assembled procapsids, but are composed exclusively of coat and scaffolding proteins

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(Fuller and King, 1982). The kinetics of in vitro procapsid assembly have been extensively studied (Prevelige et al., 1993; Teschke, 1999; Teschke et al., 2003). The nucleation complex has been proposed to comprise ~5 molecules of coat protein and 2–3 molecules of scaffolding protein (Prevelige et al., 1993). Assembly of the procapsid continues by the addition of coat protein monomers, along with scaffolding protein, to the growing edge of the new structure. Additionally, the observed self-association of P22 scaffolding protein has been suggested to aid in the conformational switching of the coat protein subunits into their proper conformation (Parker and Prevelige, 1998; Parker et al., 1997).

Scaffolding protein also plays a crucial role in formation of the correct assembly product. In vivo, it is possible for coat protein to assemble without scaffolding protein into procapsids of the correct size, although at a much decreased rate (Casjens and King, 1974; Earnshaw and King, 1978; Thuman-Commike et al., 1998). In addition, P22 coat protein can assemble without scaffolding protein in vivo into smaller $T = 4$ procapsids, as well as aberrant spiral structures (Earnshaw and King, 1978; Thuman-Commike et al., 1998). None of the structures formed in the absence of scaffolding protein are able to incorporate portal or pilot proteins (Earnshaw and King, 1978), suggesting additional roles for scaffolding protein that are essential for proper procapsid assembly and phage infectivity. A study by Parker and Prevelige (1998) showed that a buffer with minimal salt (~25 mM NaCl) allowed scaffolding and coat protein to assemble into a procapsid in vitro, while a high concentration of salt (1 M NaCl) inhibited assembly. NaCl likely has such a large effect due to the high number of positively charged residues in the carboxyl terminus of scaffolding protein, previously shown to be the coat protein binding domain (Parker et al., 1998). Our studies demonstrate that the concentration and the type of salt in the buffer are important for forming a correctly assembled procapsid of bacteriophage P22. The observed salt dependence of procapsid assembly is consistent with an electrostatic association between coat protein and scaffolding protein.

Results

To assemble a P22 procapsid in vitro, all that is necessary is monomeric coat protein and scaffolding protein. The proteins still assemble into a structure that lacks other procapsid protein components such as the portal protein complex, gp7, gp16, and gp20 but maintains similar size and morphology as compared to procapsids formed in vivo (Lenk et al., 1975). For simplicity, we will refer to these in vitro assembled procapsid-like particles as "procapsids" throughout this study. We observed that procapsids assembled in vitro in 20 mM sodium phosphate buffer, pH 7.6, migrated more slowly on an agarose gel than procapsids assembled in vivo. However, in previous experiments using a common assembly buffer, Buffer B (50 mM Tris–HCl, 25 mM NaCl, 2 mM EDTA, pH 7.6), in vitro assembled procapsids ran in the same position as in vivo assembled procapsids (Galisteo et al., 1995). From these observations, we reasoned that buffer components might be the crucial difference in the in vitro assembly of procapsids.

The effect of NaCl on assembly

To determine if increasing the ionic strength could cause in vivo and in vitro assembled procapsids to migrate in the same position on an agarose gel, various concentrations of NaCl were added to 20 mM sodium phosphate buffer. Scaffolding protein was mixed with NaCl (0–100 mM final concentration) and assembly was initiated by the addition of monomeric coat protein. In the presence of ~15 mM NaCl, in vivo and in vitro assembled procapsids migrated to the same position on an agarose gel (Fig. 1). Parker and Prevelige (1998) showed that in Buffer B in vitro assembly was efficient, while the addition of 1 M NaCl inhibited assembly. We also observed decreased levels of productive assembly at much lower NaCl conditions (>100 mM). Thus, the addition of small amounts of NaCl to 20 mM sodium phosphate buffer can lead to formation of procapsids in vitro that migrate in the same position on the agarose gel as procapsids assembled in vivo.

Lack of additional NaCl leads to aborted procapsid assembly

Negative stain electron microscopy was used to visualize the assembly products formed in the absence of NaCl to determine what structures had assembled. Samples of in vivo assembled procapsids were compared to the products of in vitro assembly reactions done with and without ~60 mM NaCl. The in vivo assembled procapsids looked typical of procapsids and were similar in diameter (Figs. 2A and B) (Teschke et al., 2003; Thuman-Commike et al., 1998, 1999).

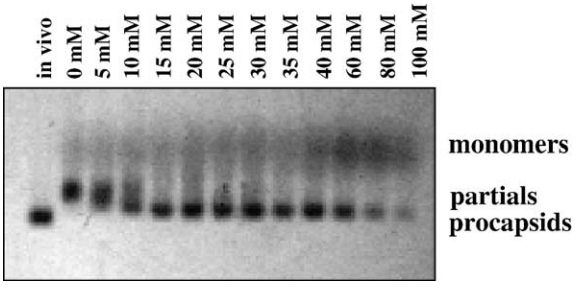


Fig. 1. NaCl alters the migration of in vitro assembled procapsids on an agarose gel. Procapsids assembled in vitro in the presence of various concentrations of NaCl at 20 °C were run on a 1.2% agarose gel. A sample of procapsids assembled in vivo was used to confirm which concentrations of NaCl allowed for proper procapsid assembly and migration. Partial procapsids migrate slower than complete procapsids. The positions of each are indicated on the right side of the figure.

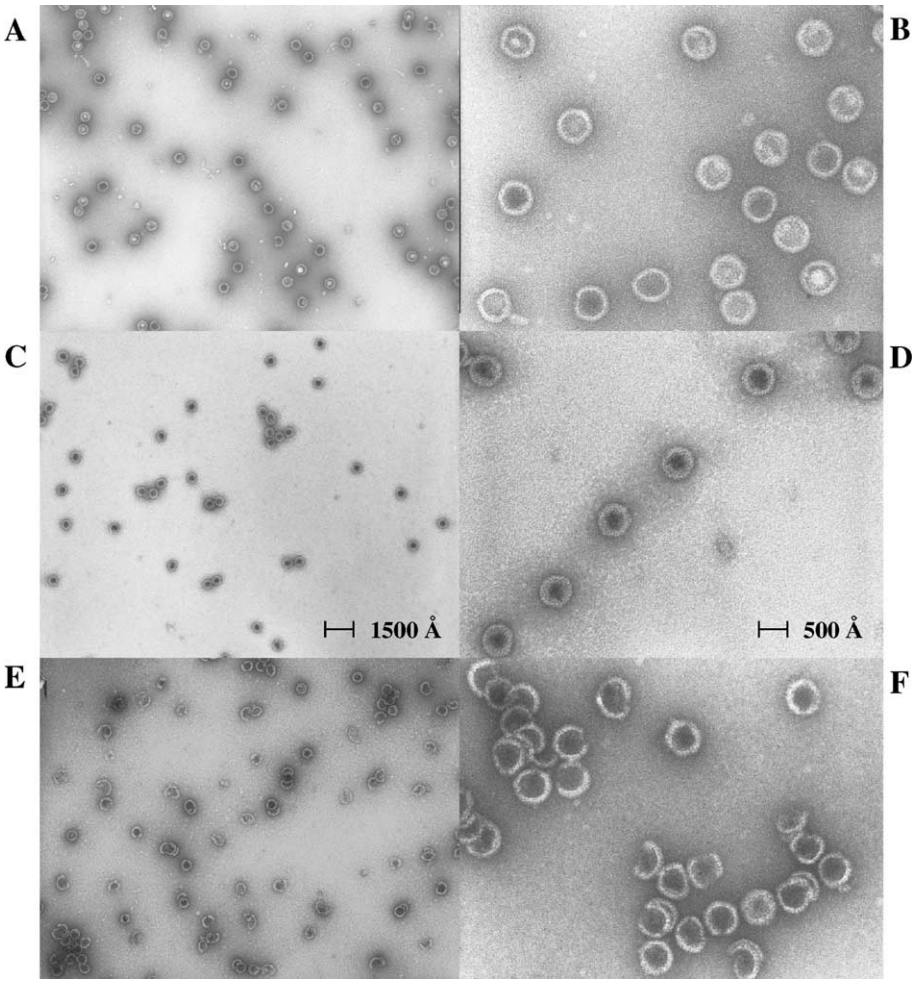


Fig. 2. Procapsid assembly is incomplete in the absence of NaCl. Negative-stain electron micrographs of in vivo assembled procapsids (A and B), procapsids assembled in vitro in the presence of 60 mM NaCl (C and D), and procapsids assembled in vitro in the absence of additional NaCl are shown (E and F). The magnifications shown are 40,000 (A, C, and E) and 126,000 (B, D, and F). Micrographs were obtained from multiple experiments; the micrographs shown are a representative sample.

The procapsids assembled in vitro in the presence of ~60 mM NaCl are shown in Figs. 2C and D. These procapsids were similar in size and shape to the in vivo assembled procapsids and also to procapsids assembled in Buffer B (Fuller and King, 1982; Prevelige et al., 1993). The procapsids assembled in the absence of NaCl (Figs. 2E and F) formed aberrant structures, leading to the formation of what appeared to be bowl-like partial capsids and other incomplete structures. In general, these partial structures had a diameter and curvature similar to those of correctly assembled procapsids. $T = 4$ procapsids were not observed in this reaction.

The partial structures appear as a somewhat heterogeneous population. Both independent partial structures and partial structures associated with one another were observed. The associated units appear as normal-sized procapsids with gaps at opposite poles, most likely due to pairing of two incomplete structures. The independent units appear to have two distinct forms: some look to be complete structures, and some appear to be bowl-like partial structures. We quantified the number of complete and partial structures in the micrographs. For our quantification, we counted all the particles visualized on multiple micrographs, from multiple wells. If the mixture was a homogeneous solution of half procapsids, the appearance of different structures can be explained by the particles assuming different positions when landing on a grid. For example, if the half particles fall on their side, they would appear as half structures from our top-down view. The structures that appear complete may actually be a half structure with the open side facing the grid or the air.

Quantification of the number of partial and complete particles was used to examine the distribution of the particles on several EM grids. In our micrographs of the assembly reactions done without NaCl, ~76% of the population appeared as partial structures. Conversely, when assembly reactions were done in the presence of salt, ~93% of the structures were complete, and partial particles were rare or absent. About 2 times more particles were produced in the assembly reactions without NaCl. This observation

was confirmed by quantification of sucrose density gradients done on the assembly reactions, discussed below. The combination of these data indicates that most of the structures seen in the absence of NaCl are actually partial particles.

The bowl-like partial capsids are able to release subunits

Sucrose gradient sedimentation was used to characterize the protein composition of the particles formed with and without NaCl. A portion of each assembly reaction, done in 20 mM sodium phosphate buffer with and without the addition of ~60 mM NaCl, was sedimented through 5–20% linear sucrose gradients. Fractions from each gradient were run on an SDS-polyacrylamide gel and quantified by densitometry (Fig. 3). The sucrose gradient of the assembly reaction done in the presence of ~60 mM NaCl showed assembled structures that sedimented to the same position as in vivo assembled procapsids (Fig. 3A). Fractions 16–22 had a reasonable coat protein:scaffolding protein ratio for in vitro assembled procapsids (~2:1) (King et al., 1973, 1978). The sample assembled in the absence of NaCl (Fig. 3B) ran much slower in the gradient than procapsids, suggesting that these assembled structures were smaller than normal procapsids, consistent with the partial structures seen in the micrographs. Moreover, fractions 12–19 had a much different coat protein:scaffolding protein ratio, ~5:1, and the scaffolding protein tailed up the gradient toward the fractions with monomeric proteins. Since the total amount of coat protein in each peak is roughly the same, there must be about twice as many partial structures as complete procapsids, which is consistent with our EM data.

The altered coat:scaffolding protein ratio seen in gradient B could be due to either the preferential release of scaffolding protein from the particles, or due to release of both coat protein and scaffolding protein. Therefore, a sample of an assembly reaction done without NaCl was diluted 10-fold before being applied to the gradient and is shown in Fig. 3C. In this gradient, there was an upward shift in the coat protein peak, as well as a slight increase in the amount of monomeric coat protein; the ratio of coat: scaffolding protein in the peak was ~11:1. The combination of these data indicates that the partial particles formed in the absence of NaCl are able to release both coat and scaffolding subunits. However, scaffolding protein binds to the partial capsids less tightly than coat protein. As a result, scaffolding protein more readily dissociates from the partial structures than coat protein.

Rapid pellet/supernatant separations were performed to demonstrate that scaffolding protein and coat protein co-migrated in the sucrose gradient as a result of a direct association with assembled structures. When pellet/supernatant separations were performed on either coat protein or scaffolding protein separately, all of the protein remained in the supernatant, indicating they are soluble and small. Conversely, when pellet/supernatant separations were performed on samples from the assembly reactions with and without NaCl, both coat protein and scaffolding protein were found in the pellet of both samples (data not shown). The combination of these data indicates that without NaCl,

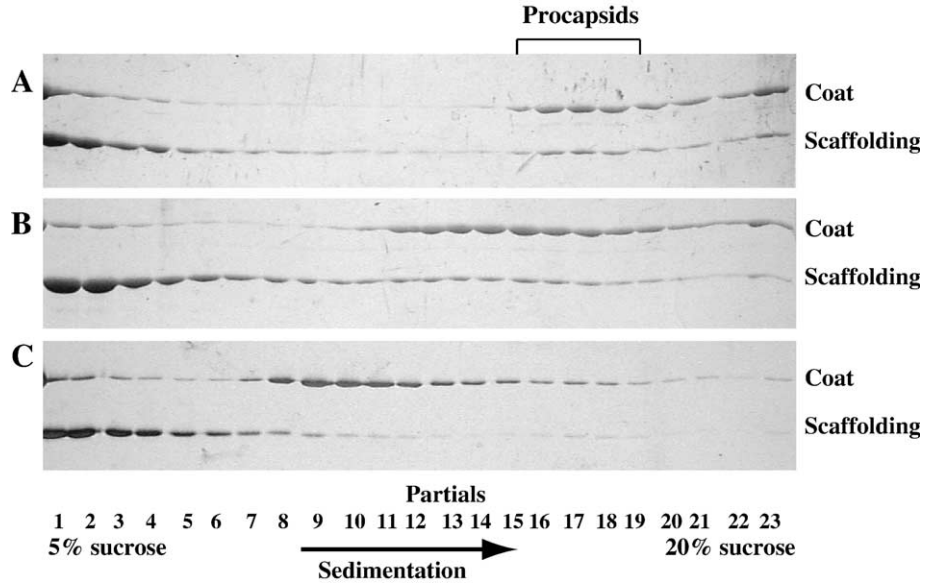


Fig. 3. Partial capsids are able to release coat protein and scaffolding protein. Samples from the assembly reactions described in the Materials and methods were sedimented through 5–20% linear sucrose gradients. Each gradient was fractionated from the top and run on 10% SDS-polyacrylamide gels. Panel A shows assembly reactions done in the presence of 60 mM NaCl and panel B shows assembly reactions done in the absence of 60 mM NaCl. The position of both coat and scaffolding protein is indicated. The position on the gradients where in vivo assembled procapsids sediment is also indicated. Panel C shows assembly reactions done in the absence of additional NaCl, but diluted 1:10 with 20 mM sodium phosphate buffer after the reaction was complete. Monomeric coat and scaffolding proteins sediment approximately to fractions 1–6. The gels in panels A and B are stained with Coomassie Blue while the gel in panel C was silver stained.

scaffolding protein interacts with coat protein to assemble into a structure smaller than normal procapsids.

Velocity sedimentation analytical ultracentrifugation experiments were done to determine the heterogeneity of species in our assembly reactions. We compared procapsids assembled in vivo, procapsids formed in vitro in the presence of ~60 mM NaCl, and the incomplete structures formed in vitro in the absence of NaCl. The *S*-values obtained for procapsids formed in vivo and procapsids assembled in the presence of NaCl were similar, with less than 2% difference between peaks, corresponding to a weight average *S*-value of 210. Conversely, the AU profiles for the particles formed in vitro in the absence of NaCl showed a broad distribution between 50 and 115 *S*, which indicates a heterogeneity of structures smaller than the size of a complete procapsid. The distribution is likely due to the different total amounts of coat and scaffolding proteins found in the partial capsids. Since such a broad profile was observed for the partial structures, they are not a monodisperse sample where all particles contain a specific number of subunits, but rather an ensemble of particles.

The formation of partial capsids is reversible

Since coat and scaffolding protein subunits are capable of being released from the partial capsids, we hypothesized that the formation of partial structures might be reversible by the addition of NaCl. To determine the reversibility of the partial capsids, the time of NaCl addition was varied relative to the initiation of the assembly reaction with coat protein. NaCl was added to scaffolding protein concomitantly with the coat protein, or at specific intervals, from 1–3000 s, after assembly was initiated. The samples were incubated for an additional 2 h, and the samples were run on an agarose gel. In all cases, the product of assembly with NaCl migrated to the same position as procapsids assembled in vivo (Fig. 4A). Assembly was also initiated in the absence of salt, and allowed to proceed for 3 days at 20 °C. This sample was then split; one aliquot was left unchanged, and 60 mM NaCl was added to the other aliquot. The samples were incubated for an additional 2 h, and the samples were run on an agarose gel. Partial particles formed in the absence of salt, as expected, while complete procapsids formed in the presence of NaCl, regardless of the time of addition. These results indicate that formation of partial structures is reversible. The partial structures formed without NaCl were found to be stable at room temperature for weeks if no NaCl was added (data not shown). These data indicate that the partial capsids are metastable, but that the reaction can be easily shifted to complete procapsid assembly.

The edge of the partial structure is competent for assembly

The observation of partial capsid structures leads to the question of why assembly stops at the partial structure. Two possibilities to consider are that the edge of the growing

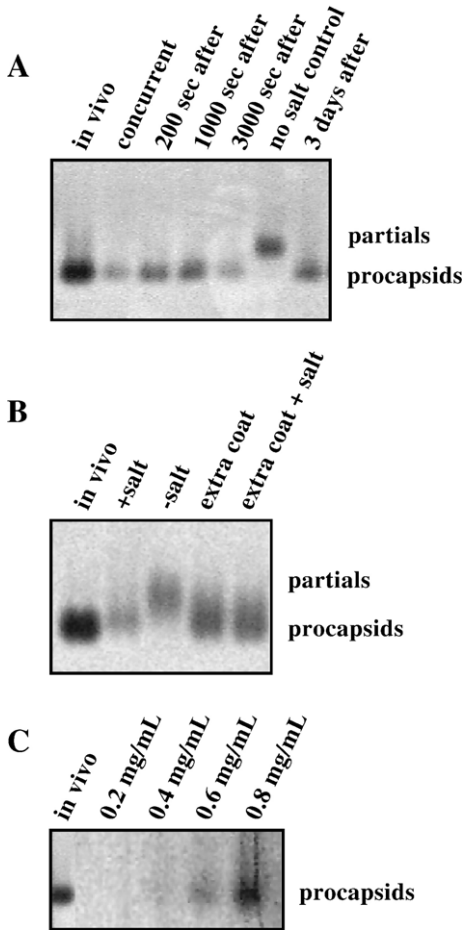


Fig. 4. Bowl-like partial capsids form because of over-nucleation. (A) NaCl was added either concurrently as the addition of coat protein or from 200 s to 3 days after the initiation of the assembly reaction. An assembly reaction done in the absence of salt is shown as a control. The position of migration of complete procapsids and partial capsids is indicated. (B) In the assembly reactions with NaCl added, a final concentration of ~60 mM NaCl was used. Both lanes labeled extra coat began as assembly reactions without NaCl. The extra coat protein, or coat protein with NaCl, was added after incubating the initial assembly reactions for 2 h; the reactions were assembled for a total of 4 h. (C) Assembly reactions with no NaCl were initiated with scaffolding protein held constant (0.25 mg/mL, ~7.5 μM) and varying amounts of coat protein were added (0.2–0.8 mg/mL, ~4–21 μM). The reactions were allowed to proceed to 2 h at 20 °C. Reproduction of the gel was difficult due to low intensity bands at such low protein concentrations, however, the presence of procapsids was clearly visualized by the eye for coat protein concentrations greater than 0.2 mg/mL. The assembly reactions were run on 1.2% agarose gels and visualized with Coomassie blue.

procapsid becomes incompetent for the addition of coat protein subunits when assembled in the absence of NaCl or that the coat protein concentration becomes limiting to the reaction. To determine if coat protein becomes limiting in reactions without salt, in vitro assembly reactions were done as previously described. A reaction without NaCl was split into three aliquots: no addition of salt or coat protein, addition of extra coat protein monomers, and addition of extra coat protein monomers plus NaCl (~60 mM final). All of the reactions were incubated for an additional 2 h before

the samples were applied to the agarose gel (Fig. 4B). The structures formed in the assembly reactions both with and without NaCl migrated on the agarose gel. The assembly reaction with additional coat protein and the reaction with additional coat protein plus NaCl yielded structures that migrated to the same position as the in vivo assembled procapsids. These data indicate that the edge of the procapsid is competent for assembly, and with additional coat protein, complete procapsids can be formed. Therefore, we hypothesize that the absence of NaCl may cause the formation of more nuclei compared to the number created in the presence of NaCl. Ultimately, this would cause the coat protein concentration to become limiting for the reaction, resulting in formation of partial structures.

Complete capsids form when scaffolding protein is limiting

To determine if coat protein is limiting due to an increased number of nuclei, a series of titrations were performed. In these reactions, scaffolding protein was held constant at a limiting concentration in the absence of salt, and coat protein levels were increased. Varying concentrations of coat protein (4–21 μM, 0.2–0.8 mg/mL) were added to initiate assembly with very low scaffolding protein concentrations (7.5 μM, 0.25 mg/mL). The samples were then run on an agarose gel. When coat protein was added at 0.2 mg/mL no assembly occurred, as this is below the critical concentration for assembly (Prevelige et al., 1988; Teschke and Fong, 1996). For all other concentrations of coat protein, the bands on the agarose gel migrated to the same position as complete procapsids (Fig. 4C). These data confirm that coat protein is the limiting factor when formation of partial capsids is observed. In total, our data indicate that the absence of NaCl causes formation of too many nuclei.

Formation of partial structures results in altered assembly kinetics

Since the electron micrographs indicated that the assembly reactions led to different products in the presence or absence of ~60 mM NaCl, we asked if the kinetics of the reactions would be different as well. Assembly reactions were performed, with and without ~60 mM NaCl added to scaffolding protein prior to the initiation of assembly by the addition of coat protein. The kinetics of assembly were monitored by light scattering at 500 nm in a fluorometer (Fig. 5). In the presence of NaCl, procapsid assembly progressed as previously observed in Buffer B (Prevelige et al., 1993). A short lag phase (~20–30 s) preceded a rapid increase in light scattering as the procapsids were assembled (Fig. 5A). In the absence of NaCl, no initial lag phase was observed (Fig. 5B). The slope was determined for four pairs of assembly reactions done on different days to compare the rate of the elongation phases in the presence and absence of NaCl. The elongation phase was ~2.4 ± 0.8 times faster

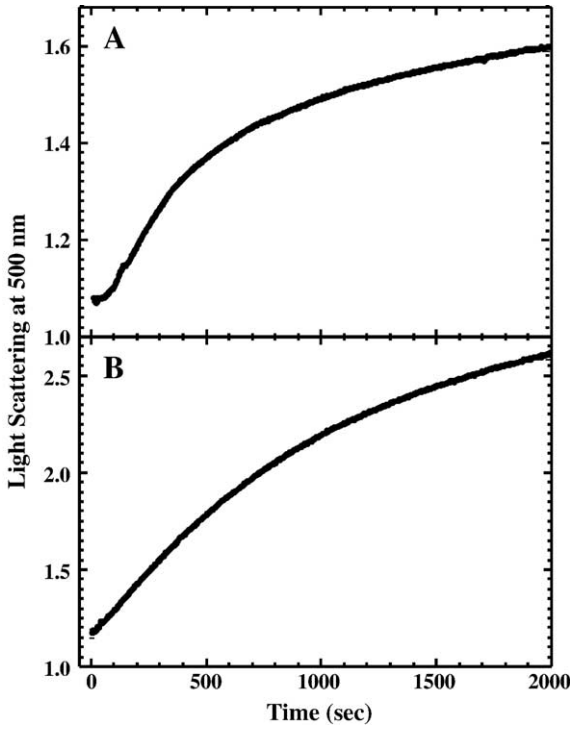
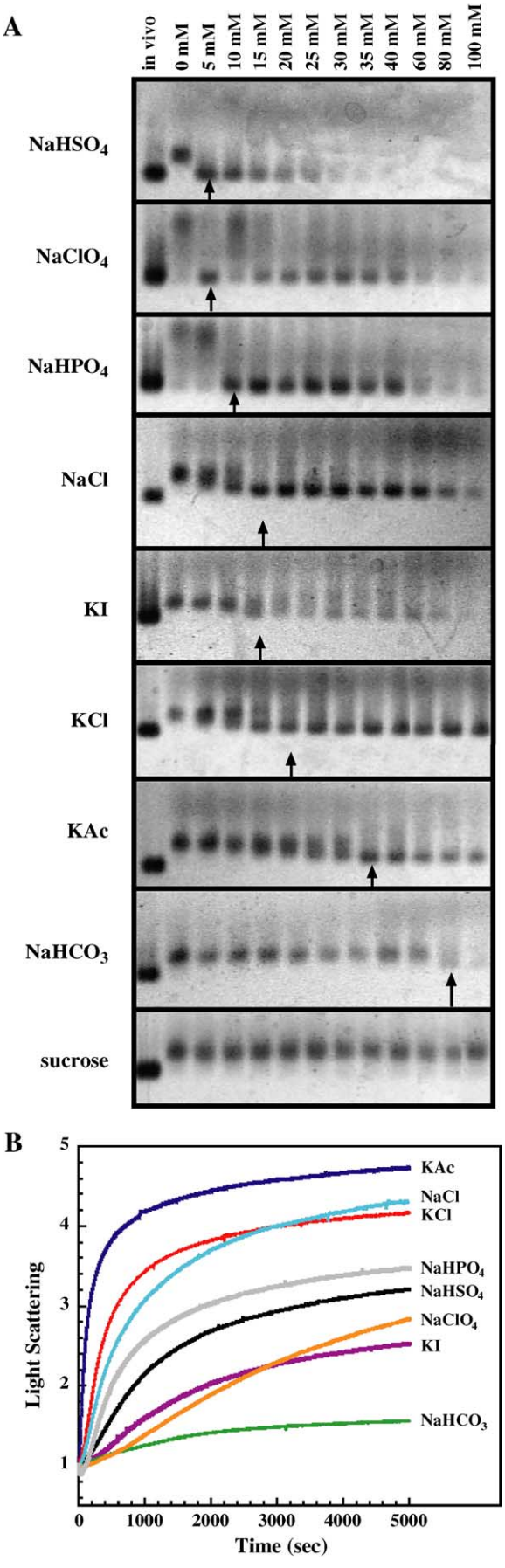


Fig. 5. The assembly reactions proceed differently in the presence and absence of NaCl. Purified coat (0.63 mg/mL final, ~13 μM) and scaffolding (0.58 mg/mL final, ~18 μM) proteins were mixed in a cuvette in the presence of either 20 mM phosphate buffer with 60 mM NaCl (A) or in 20 mM phosphate buffer without additional NaCl (B). The reactions were monitored by light scattering in a fluorometer with the excitation and emission wavelengths set to 500 nm.

when NaCl was present compared to the elongation phase in the absence of salt.

Procapsid formation occurs with a salt dependence that approximately follows the electroselectivity series for anions

NaCl clearly affects procapsid assembly. In order to determine if a specific salt is critical for promoting proper procapsid assembly in vitro, assembly reactions were performed using varied concentrations of potassium chloride (KCl), potassium acetate (KAc), sodium perchlorate ($NaClO_4$), sodium phosphate ($NaHPO_4$), potassium iodide (KI), sodium carbonate ($NaCO_3$), and sodium sulfate ($NaHSO_4$). Samples were then run on agarose gels to visualize the assembly products. In vitro assembled procapsids that migrated to the same position as procapsids assembled in vivo were observed for all the salts tested, but proper procapsid formation occurred at different concentrations depending on the salt (Fig. 6A). Sucrose, an osmolyte (Bolen and Baskakov, 2001), was also added to assembly reactions and was unable to promote correct procapsid assembly, suggesting that formation of complete procapsids is dependent on salt addition and not the osmotic strength of the solution (Fig. 6A). Glycerol also had no effect on correct procapsid assembly (data not shown). The effect of the salts on procapsid assembly appears to



approximately follow the electroselectivity series for anions, which typically indicates that ionic interactions are involved in protein association reactions (Gjerde et al., 1980; Gregor et al., 1955).

In order to determine if low concentrations of salt were able to shift the reaction products from partial capsids to complete capsids, samples of all of the assembly reactions shown in Fig. 5A were aged for 2 weeks at 4 °C. In the absence of salt, bands migrated to the same position as the partial capsids, indicating that these partial structures are stable for long periods of time. For all the other samples ranging from 5–100 mM salt, the structures migrated in the same position as procapsids, given a long enough incubation time (data not shown). These data indicate that the reaction products can be shifted from partial to complete structures in the presence of low salt concentrations.

The kinetics of assembly were different with and without addition of NaCl (Fig. 5). Since the conditions for proper assembly appear to be dependent on the specific type of salt added (Fig. 6A), we wanted to ascertain if the kinetics of assembly would differ as well. Therefore, assembly reactions were done in the presence of KCl, KAc, $NaClO_4$, $NaHPO_4$, KI, $NaHCO_3$, and $NaHSO_4$ to determine whether or not the specific salt changed the rate of assembly. The salts were added at the minimum concentration required to form complete procapsids (arrows in Fig. 6A). Light scattering was used to monitor assembly, and the data are shown in Fig. 6B. A correlation was not observed between the rate of assembly and the concentration of each salt required to form complete procapsids. The different salts caused both a change in lag time, as well as the yield of product formation. The light scattering intensity is consistent with the agarose gels, shown in Fig. 6A, which reveal different amounts of complete procapsids formed in the presence of different salts. Since no correlation was observed between the order of hydration for salts in the agarose gels and the assembly kinetics, we analyzed the kinetics based on the chaotropic (order-breakers) and kosmotropic (order-makers) effects of the anions. The chaotropes, iodide and perchlorate, showed a decrease in rate and extent of procapsid formation. Conversely, the kosmotropes, phosphate, sulfate, and acetate,

Fig. 6. Proper procapsid formation depends on salts approximately following the electroselectivity series for anions. (A) Procapsids assembled in vitro in the presence of various salts, ranging from 0–100 mM, were run on a 1.2% agarose gel and are shown. A sample of procapsids assembled in vivo was used to confirm the concentrations of salt required for proper procapsid assembly and migration. The arrows indicate the minimum concentration of salt where discrete procapsids are formed. These titrations were repeated three times for each salt. Similar results were determined from each experiment. (B) Purified coat (0.6 mg/mL final, ~13 μM) and scaffolding (0.6 mg/mL final, ~18 μM) proteins were mixed in a cuvette in the presence of 20 mM sodium phosphate buffer with the addition of various salts. The light scattering of each reaction was monitored at 500 nm in a fluorometer. Salt concentrations used were the minimal amount required to form procapsids, based on the data shown in Fig. 4 (5 mM for $NaClO_4$, 5 mM for $NaHSO_4$, 10 mM for $NaHPO_4$, 15 mM for NaCl, 15 mM for KI, 20 mM for KCl, 35 mM for KAc, and 80 mM for $NaHCO_3$).

resulted in an increase in rate and yield. Chloride, which is neither a chaotrope nor kosmotrope (Collins, 1997), fell midway in the kinetic series. In total, these data indicate that chaotropic anions decrease product formation, whereas kosmotropic anions contribute to procapsid formation.

NaCl effects the secondary structure of coat protein

Salts affect the interaction between coat and scaffolding proteins. To determine if there were conformational changes, intrinsic tryptophan fluorescence and circular dichroism (CD) wave scans were done with both coat and scaffolding proteins, in the presence and absence of 60 mM NaCl. The fluorescence emission maximum and shape of the fluorescence wave scans were unchanged for either protein upon the addition of salt (Figs. 7A and B). The slight change in fluorescence intensity of scaffolding protein is likely a result of small variations in final protein concentration, and not due to an altered structure. The CD spectrum of scaffolding protein also remained unchanged with the addition of salt (Fig. 7C). However, the CD spectrum of coat protein (Fig. 7D) was altered in the presence of 60 mM NaCl, particularly observed by a decrease in negative ellipticity between 200 and 230 nm. These data suggest that the addition of 60 mM NaCl alters the secondary structure of coat protein.

Discussion

The coat protein binding region of scaffolding protein has a high percentage of positively charged amino acids. As a result, the interactions between coat and scaffolding proteins are likely electrostatic (Parker and Prevelige, 1998). Prevelige's group has demonstrated that high salt concentrations compromise the ability of scaffolding protein to interact with intact procapsid shells. Our observations suggest that salt promotes a change in the coat protein: scaffolding protein interaction early in the association reaction, and that ionic interactions regulate correct assembly of procapsids.

Our data provide evidence that salts affect the association of protein subunits both in the nucleation and the elongation reactions. Without, salt too many nuclei form, resulting in bowl-like partial capsids. Therefore, we propose that increasing the concentration of salt decreases the affinity of the interaction between coat protein and scaffolding protein, and that nucleation is regulated by ionic interaction.

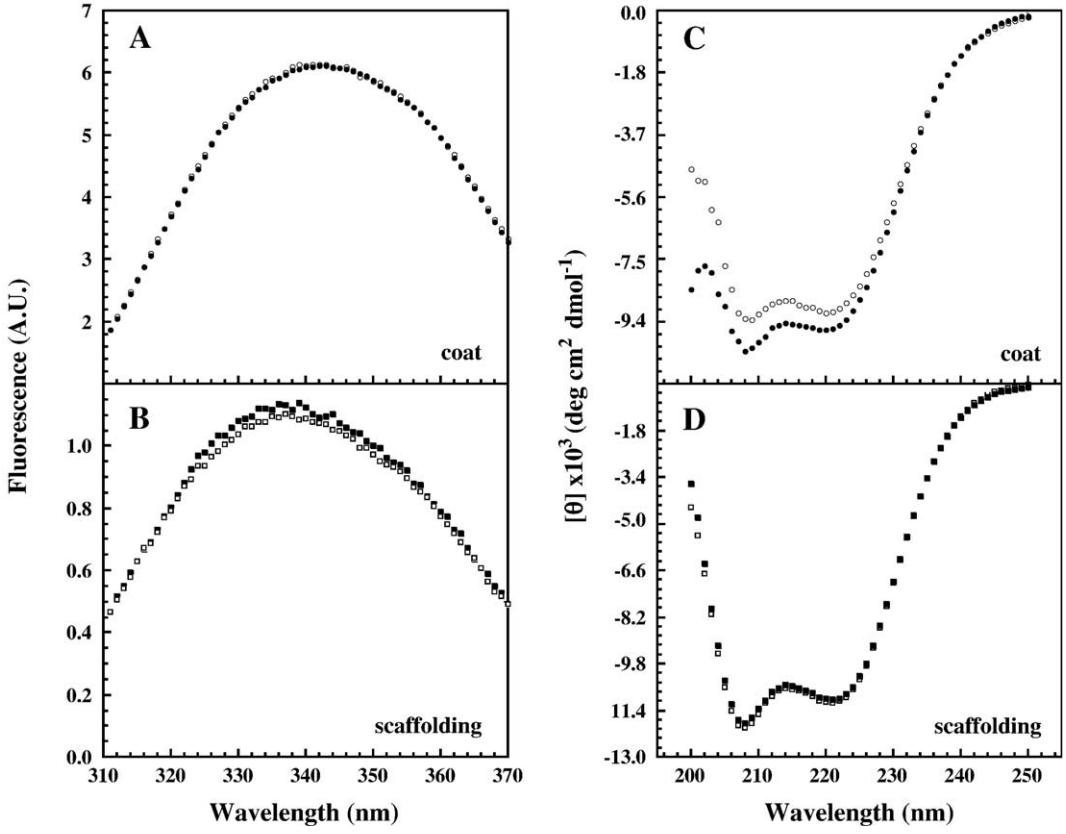


Fig. 7. NaCl effects secondary structure of coat protein. Circular dichroism and fluorescence spectra of monomeric coat and scaffolding proteins were taken at 20 °C as described in the Materials and methods. Closed symbols represent samples in 20 mM sodium phosphate buffer; open symbols represent the samples in 20 mM sodium phosphate buffer with the addition of 60 mM NaCl. All samples were equilibrated at 20 °C for 1 h before scanning. This experiment was repeated three times with similar results.

In addition, different salts change the rate of elongation, suggesting a role for ionic interactions in this step as well.

The composition of the buffer alters assembly of P22 procapsids

Our results show that assembly is affected by both salt concentration and salt type, approximately following the electroselectivity series for anions (Gjerde et al., 1980; Gregor et al., 1955). This behavior is generally indicative of changes in electrostatic interactions, suggesting a role for weak ionic interactions between the subunits. We hypothesize that P22 capsid assembly is driven by multiple, specific, weak protein:protein interactions of viral subunits during the assembly process. Zlotnick (2003) has proposed that weak protein:protein interactions are a common feature of viral capsids. An abbreviated version of the electoselectivity series is shown below:

Strong						Weak
SO_4^{2-}	> ClO_4^-	> I^-	> Cl^-	> HCO_3	> HPO_4^{2-}	> CH_3COO^-

Order of anions affecting P22 assembly:

Strong						Weak
SO_4^{2-}	= ClO_4^-	> HPO_4^{2-}	> I^-	= Cl^-	> CH_3COO^-	> HCO_3

For P22 procapsid assembly, the addition of strongly hydrated salts causes assembly to occur more rapidly; however, a greater amount of salt is required to shift the assembly reaction from partial to complete capsid production. We believe that anions are responsible for this effect since NaCl, KCl, and Mg_2Cl (not shown) produced similar results. This effect was also observed for phosphate salts, where sodium and potassium reacted the same. Although phosphate and bicarbonate are outliers in the electroselectivity series for our system, we believe anionic interactions are necessary and important for mediating P22 procapsid assembly. Anions could either alter formation of salt bridges between coat and scaffolding proteins or change solvent hydration of the individual proteins. We favor that increasing the anion concentration decreases the affinity for coat and scaffolding interactions, regulating the number of nuclei. Only the conformation of coat protein is affected by NaCl; therefore, solvent effects seem less likely. Additionally, over nucleation in the absence of NaCl can be overcome simply by the addition of extra coat protein, which indicates that NaCl is not required for coat protein to be competent for assembly.

How salts might affect assembly

The association rate of protein–protein interactions is often sensitive to ionic composition while the off-rate is only weakly affected (Escobar et al., 1993; Radic et al., 1997; Schreiber and Fersht, 1993; Wallis et al., 1995; Wendt et al., 1997; Zhou, 2001). If the association constant (K_a) of the coat protein:scaffolding protein interaction is increased without a compensating decrease in protein concentration, a kinetic trap could occur. In this case, too many nuclei would form and there would not be enough free monomeric protein for procapsid assembly, which would lead to incomplete capsid formation (Zlotnick, 1994; Zlotnick et al., 1999, 2000). A study using the $T = 3$ cowpea chlorotic mottle virus showed that when the pentameric nucleation complex formed too rapidly, the concentration of the free dimeric subunit was depleted. This resulted in the formation of aberrant pseudo-$T = 2$ structures from the rapidly formed pentamers of dimers (Zlotnick et al., 2000). These results correspond well with what we observe for assembly at low salt concentrations. The association rate of scaffolding protein and coat protein is likely sensitive to the ionic composition; in the absence of salt, many partial procapsids are formed and most of the monomeric coat protein has been incorporated into these structures.

A model for control of assembly of P22 procapsids

Previous models were used to understand P22 procapsid assembly that were based on assembly of filamentous proteins (Prevelige et al., 1993). In filamentous assembly, there is one nucleus for a very long polymer. (Oosawa and Asakura, 1975). In contrast, for an icosahedral particle such as a phage P22 procapsid, there are a specific number of subunits per particle based on the T number of the virus. The observed assembly kinetics are a direct result of the time it takes to complete a discrete particle (Endres and Zlotnick, 2002; Zlotnick et al., 1999; Zlotnick, 1994). During the lag phase of icosahedral virus assembly reactions, intermediates accumulate until each intermediate reaches a steady-state concentration (Fig. 8A). Free subunits can then interact with the intermediates, resulting in a rapid increase in complete particles, which is observed during the elongation phase. Eventually, the concentration of free subunits is depleted, resulting in a decrease in rate as a slow asymptotic approach to equilibrium (Fig. 8A) (Endres and Zlotnick, 2002; Zlotnick, 1994). If association of the subunits starts at too many points, the reaction may stall at a “kinetic trap”, resulting in partial formed particles. Control of the nucleation step to limit the number of starting points is one way to avoid such a kinetic trap (Johnson et al., 2005).

Alterations in the secondary structure of coat protein upon addition of NaCl were observed; therefore, the small changes in coat protein structure likely affect the interaction between coat and scaffolding proteins. Fig. 8B describes the association reaction between coat and scaffolding protein monomers in the absence of salt. In reaction 1, the association of coat and scaffolding protein occurs with a higher than normal affinity. This leads to the formation of too many nuclei, which in turn depletes the population of monomeric coat proteins, ultimately causing the formation of incomplete procapsid formation. Our data suggest that these

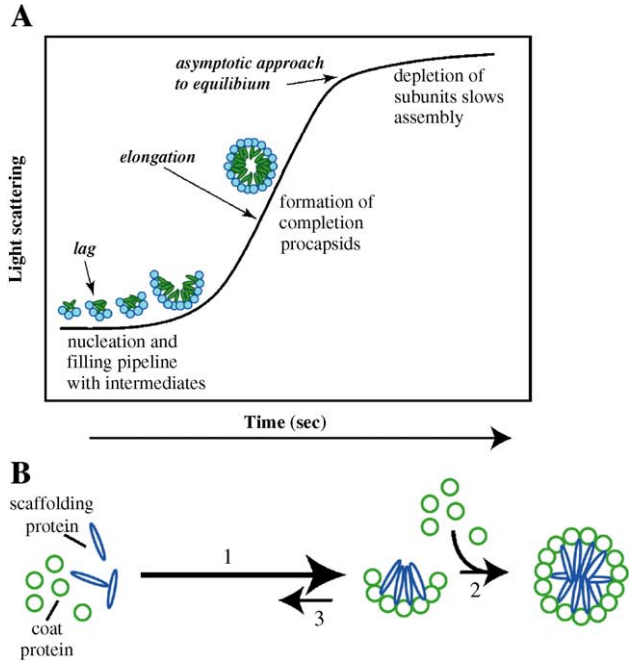


Fig. 8. Model of the process of viral assembly. See Discussion for a complete description of this figure.

incomplete, bowl-like partial capsids are metastable, indicating the relative rates of the back reaction to subunits (shown in reaction 3) and the forward progression to procapsids (shown in reaction 2) are slow in comparison to the forward rate of reaction 1 in low salt solutions. Thus, our data support a role for multiple weak, interactions controlling self-assembly of procapsids; ionic interactions are critical to control nucleation. Perhaps the regulation of nucleation can be exploited for development of anti-viral drugs.

Materials and methods

Chemicals, buffers, and proteins

Ultrapure urea was purchased from ICN. All other chemicals were reagent grade and purchased from common sources. Purification of coat protein was done as previously described (Galisteo et al., 1995; Prevelige et al., 1988; Teschke, 1999; Teschke and King, 1993). All experiments described below were done in 20 mM sodium phosphate buffer made using Na_2HPO_4, with the pH adjusted to 7.6 with H_3PO_4, and with the addition of various salts as indicated. The pH of the salt solutions was adjusted to pH 7.6, where needed.

Refolded coat protein monomers

Coat protein monomers were obtained from urea-denatured empty procapsid shells, as described previously, using dialysis at 4 °C against 20 mM sodium phosphate buffer, pH 7.6 (Anderson and Teschke, 2003; Doyle et al., 2004; Doyle et al., 2003). Coat protein monomers obtained in this way are assembly-competent but will not assemble into procapsids until the addition of scaffolding protein (Fuller and King, 1981, 1982; Prevelige et al., 1988; Teschke, 1999; Teschke and King, 1993, 1995).

Agarose electrophoresis

Agarose gels were prepared and run as previously described (Lanman et al., 1999; Serwer and Pichler, 1978; Teschke et al., 2003; Tuma et al., 1998). In brief, the samples for the native agarose gel were prepared by combining a portion of the protein with agarose gel sample buffer and ~5 μg was loaded onto 1.2% Seakem HGT agarose gel. The gels were run at 17 V constant for ~24 h at 4 °C. The agarose gels were stained with Coomassie Blue.

Assembly reactions in the presence of NaCl

To assemble coat protein into procapsids in vitro, refolded coat protein monomers at a final concentration of 0.56 mg/mL (~12 μM) were mixed with scaffolding protein at a final concentration of 0.67 mg/mL (~20 μM) at 20 °C in a total volume of 50 μL. Various final concentrations of NaCl, between 0 and 100 mM, were added to scaffolding protein prior to initiation of assembly with coat protein. Assembly reactions were incubated for 2 h at 20 °C. Samples from each reaction were run on 1.2% agarose gels as described above.

Negative stain electron microscopy

A portion of the assembly reactions as described above was used for negative stain electron microscopy (EM). The samples were spun in a microfuge at maximum speed for 5 min to remove any debris. Five microliters of the sample was allowed to absorb to the carbon-coated grid for 15 s. Two to three drops of water were used to wash the grid. The sample was stained with two to three drops of 1% aqueous uranyl acetate for 30 s. The excess liquid was wicked off and the grid air-dried. The samples were viewed using a Philips Model 300 TEM at 80 kV with a magnification of both 40,000 and 126,000.

Assembly reactions monitored by light scattering and sucrose gradients

Large assembly reactions (250 μL) monitored in a SLM Aminco-Bowman 2 spectrofluorometer were done at 20 °C. The reaction was monitored by the increase in 90° light scattering at 500 nm with the bandpasses set to 4 nm. In the experiment shown in Fig. 3, panels A and B, the final coat protein concentration was 0.6 mg/mL (~13 μM) and the final scaffolding protein concentration was 0.58 mg/mL (~18 μM). Three assembly reactions were done: (1) 60 mM NaCl final added prior to the addition of coat protein, (2) no

NaCl added, and (3) no NaCl added, the reaction was allowed to proceed for 2 h and then diluted 1:10 with 20 mM sodium phosphate buffer. After the assembly reactions were monitored for 2000 s in the fluorometer, 75 μL of each reaction mixture was applied to the top of a 2.2 mL, linear 5–20% (w/w) sucrose gradient, prepared using a BioComp Gradient Master. The gradients were centrifuged in a Sorvall RCM120EX centrifuge with an RP55S rotor for 40 min at 35,000 rpm at 20 °C. After centrifugation, the gradients were fractionated from the top into 100 μL fractions. The fractions were run on a 10% SDS-polyacrylamide gel. Reactions 1 and 2 were then Coomassie stained; reaction 3 was silver stained. The coat protein bands were quantified using a Kodak EDAS system.

Sedimentation velocity analytical ultracentrifugation (AU) experiments

Sedimentation velocity analytical ultracentrifugation experiments were performed in a Beckman XL-I AU using interference optics. Seven double sector cells were assembled with aluminum filled epon centerpieces, sapphire windows, and interference slits. The samples were centrifuged at 15,000 rpm at 20 °C until boundary sedimentation was complete. The samples studied were in vivo assembled procapsids, as well as samples assembled in vitro in 20 mM sodium phosphate buffer with the addition of 0 and 60 mM NaCl. All samples were dialyzed against their respective buffers after assembly. The data were analyzed using Sedfit C(S) analysis (Schuck, 2000).

Time of NaCl addition to assembly reaction

Small (50 μL) assembly reactions were done as described above, but the time of NaCl addition to the assembly mixture was varied. A final concentration 60 mM NaCl was added to coat protein from 0–3000 s after the initiation of assembly with coat protein. Reactions were incubated at 20 °C for 5 h. In addition, one reaction was allowed to proceed for 3 days at 20 °C. This sample was then split; one aliquot was left unchanged, and 60 mM NaCl was added to the other aliquot. The samples then incubated at 20 °C for an additional 3 h. Samples from each reaction were run on 1.2% agarose gels as described above. The final coat protein concentration was 0.53 mg/mL (~11 μM) and the final scaffolding protein concentration was 0.55 mg/mL (~17 μM).

Determining if the edge of the procapsid is competent

Assembly reactions were done as described above. The final coat protein concentration was ~0.8 mg/mL (~17 μM) and the final scaffolding protein concentration was ~0.66 mg/mL (~20 μM). A final concentration of 60 mM NaCl was added to some control reactions prior to the initiation of assembly by coat protein. The reactions were initiated by the addition of coat protein and allowed to proceed for 2 h. After 2 h, 50% more molecules of coat protein were added to one of the reaction mixtures and 50% more coat protein molecules as well as NaCl (60 mM final concentration) were added to a second reaction mixture. The reactions were incubated for an additional 2 h and run on a 1.2% agarose gel.

Determining if coat protein is limiting

To determine if coat protein is limiting, scaffolding protein was held constant at 0.25 mg/mL (~7.5 μM), and coat protein concentrations ranging from 0.2–0.8 mg/mL (~4–21 μM) were added to initiate assembly in 20 mM sodium phosphate buffer in the absence of salt. All assembly reactions were allowed to proceed for 2 h at 20 °C. These assembly reactions were run on a 1.2% agarose gel as described above.

Assembly reactions using salts according to the electroselectivity series

To assemble coat protein into procapsids in vitro, refolded coat protein monomers at a final concentration of 0.6 mg/mL (~13 μM) were mixed with scaffolding protein at a final concentration of 0.6 mg/mL (~18 μM) at 20 °C in a total volume of 50 μL. Various final concentrations of KAc, KCl, KI, NaCl, $NaHPO_4$, $NaHSO_4$ $NaClO_4$, $NaHCO_3$, sucrose, and glycerol between 0 and 100 mM were added to scaffolding protein prior to initiation of assembly with coat protein. Assembly reactions were incubated for 2 h at 20 °C. Samples from each reaction were run on 1.2% agarose gels as described above.

Kinetic experiments using various types of salt

Large assembly reactions (250 μL) monitored in a SLM Aminco-Bowman 2 spectrofluorometer were done at 20 °C. The reaction was monitored by the increase in 90° light scattering at 500 nm with the bandpasses set to 4 nm. Purified coat (0.6 mg/mL final, ~13 μM) and scaffolding (0.6 mg/mL final, ~18 μM) proteins were mixed in a cuvette in the presence of 20 mM sodium phosphate buffer with the addition of various salts. Salt concentrations were chosen based on the minimal amount required to form procapsids (5 mM for $NaClO_4$, 5 mM for $NaHSO_4$, 10 mM for $NaHPO_4$, 15 mM for NaCl, 15 mM for KI, 20 mM for KCl, 35 mM for KAc, and 80mM for $NaHCO_3$). The salts were added to scaffolding protein prior to the initiation of assembly by the addition of coat protein.

Tryptophan fluorescence emission scans

Fluorescence experiments were done with an SLM Aminco-Bowman 2 spectrofluorometer. The temperature of the cuvette was maintained at 20 °C with a circulating water bath. For emission scans, the excitation wavelength was 295 nm and the emission was scanned from 310–370 nm with a

scan rate of 0.25 nm/s. The excitation and emission bandpasses were set to 1 and 8 nm. A 1 cm pathlength cell was used. The final concentrations of both refolded coat protein monomers and scaffolding protein were 100 μg/mL.

CD wavelength scans

Circular dichroism (CD) was done with an Applied Photophysics Pi-Star 180 circular dichroism spectrapolarimeter with the cuvette maintained at 20 °C with a circulating water bath. Wavelength scans were done over 200–250 nm, sampled every 1 nm, with entrance and exit slit widths of 2 nm and a scan time of 15 min. A 1 mm pathlength cell was used for the CD wavelength scans. The final protein concentrations were 0.7 mg/mL for coat protein and 0.6 mg/mL for scaffolding protein.

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