Electrostatic Interactions Drive Scaffolding/Cloak Protein Binding and Procapsid Maturation in Bacteriophage P22

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The first step in assembly of the bacteriophage P22 is the formation of a T = 7 icosahedral "procapsid," the major components of which are the coat protein and an inner core composed of the scaffolding protein. Although not present in the mature virion, the scaffolding protein is required for procapsid assembly. Eleven amino-acid residues at the extreme carboxyl terminus of the scaffolding protein are required for binding to the coat protein, and upon deletion of these residues, approximately 20 additional residues become disordered. Sequence analysis and NMR data suggest that the 30 residues at the carboxyl terminus form a helix-loop-helix motif which is stabilized by interhelical hydrophobic interactions. This "coat protein recognition domain" presents an unusually high number of positively charged residues on one face, suggesting that electrostatic interactions between this domain and the coat protein may contribute to recognition and binding. We report here that high ionic strength (1 M NaCl) completely inhibited procapsid assembly in vitro. When scaffolding protein was added to empty procapsid "shells" of coat protein, 1 M NaCl partially inhibited the binding of scaffolding protein to the shells. This suggests that the positively charged coat protein recognition domain at the carboxyl terminus of the scaffolding protein binds to a negatively charged region on the coat protein. During DNA packaging, the scaffolding protein exits the procapsid; scaffolding protein exit is followed by the expansion of the procapsid into a mature capsid. Procapsid shells can be induced to undergo a similar expansion reaction in vitro by heating (45–70°C); this process was also inhibited by 1 M NaCl. These results are consistent with a model in which negatively charged scaffold protein-binding domains in the coat proteins move apart during procapsid expansion; this relief of electrostatic repulsion could provide a driving force for expansion and subsequent maturation. High-salt concentrations would screen this repulsion, while packaging of DNA (a polyanion) in vivo may increase the instability of the procapsid enough to trigger its expansion.

INTRODUCTION

The Salmonella typhimurium bacteriophage P22 assembles by a pathway that is typical of the double-stranded DNA (dsDNA)2 phage (Hendrix, 1985; Casjens and Hendrix, 1988) and of related viruses such as herpesviruses (Rixon, 1993; Donaghy and Jupp, 1995; Hong et al., 1996; Newcomb et al., 1996; Trus et al., 1996; Wood et al., 1997) and adenoviruses (D’Halluin et al., 1978). The initial step in P22 assembly is the copolymerization of 420 molecules of the coat protein (gp5) into a T = 7 icosahedral shell termed a "procapsid" (Casjens, 1979; Prasad et al., 1993; Thuman-Commike et al., 1996). In addition to this coat protein shell, the procapsid contains an inner core of 250 ± 100 scaffolding protein (gp8) molecules (Casjens and King, 1974; Eppler et al., 1991), a dodecameric ring of portal proteins (gp1) at one of the fivefold vertices of symmetry (Bazinet et al., 1988), and 10 ± 20 copies of each of three "pilot proteins" (gp7, gp16, and gp20) which are required for DNA injection into the host (Bryant and King, 1984; Thomas and Prevelige, 1991; Umlauf and Dreiseikelmann, 1992). Double-stranded DNA is subsequently packaged into the procapsid through the portal complex (Bazinet and King, 1985), while the scaffolding proteins exit the structure (presumably via holes which have been observed within the hexameric clusters of coat protein subunits in the procapsid lattice (Prasad et al., 1993)). The procapsid then expands in radius by about 10% and becomes more angular in appearance (Earnshaw et al., 1976). After expansion, the tailspike and other remaining proteins required for infectivity are attached (Strauss and King, 1984).

The precise mechanism by which these viruses accomplish the initial stage of assembly, the construction of the procapsid, remains unknown. This process is of great interest, both from the perspective of understanding how proteins can self-assemble into complex structures and for consideration of assembly as a potential target for antiviral drug development (Prevelige, 1998). Of the six proteins present in the P22 procapsid, only the

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2 Abbreviations used: dsDNA, double-stranded DNA; NMR, nuclear magnetic resonance spectroscopy; HSV-1, herpes simplex virus type 1; CMV, cytomegalovirus; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; GuHCl, guanidinium hydrochloride; EDTA, ethylene diamine tetraacetic acid.
coat and scaffolding proteins are required for assembly. These proteins can be purified, and when mixed together in vitro, they rapidly and efficiently assemble particles that very closely resemble procapsids (Prevelige et al., 1988). The geometry of the procapsid mandates that each of the 420 chemically identical coat protein subunits must be able to adopt one of seven quasiequivalent conformations at the seven symmetry-related positions within the procapsid (Caspar and Klug, 1962). The coat protein cannot accomplish this efficiently without the assistance of the scaffolding protein, which serves as an "assembly chaperone," both ensuring that the proper conformations of the coat protein subunits are added to the growing shells at the proper positions and greatly accelerating the process (Earnshaw and King, 1978; Fuller and King, 1981; Prevelige et al., 1988).

The P22 scaffolding protein is a 303-residue, highly elongated molecule which appears to consist of several helical sections separated by turns and random coil segments (Tuma et al., 1996). Because of its unusually flexible structure, it has not been possible to obtain crystals of this critical protein (P.E.P., unpublished results). Thus no X-ray diffraction data are available, and since the scaffolding protein is too large for NMR, its three-dimensional structure is not known. However, several truncated scaffolding proteins have been cloned, expressed, and purified. Removal of as much as 80% of the scaffolding protein can be performed without eliminating activity, as long as the carboxyl-terminal portion of the protein is retained (Parker et al., 1998; M.H.P., P. Weigele, S. Casjens, and P.E.P., unpublished results). Determination of the structure of a 66-residue scaffolding protein fragment by NMR is currently underway (Y. Sun, M.H.P., P.E.P., and N. Krishna, unpublished results).

In the absence of precise structural information, many lines of biophysical evidence have provided clues as to how the scaffolding protein functions. Analytical ultracentrifugation has revealed that the scaffolding protein forms a mixture of monomers, dimers, and tetramers at physiologically relevant concentrations. A mutant (R74C/L177I) containing a single cysteine residue forms disulfide-crosslinked covalent dimers and is more active than the wild-type protein, suggesting that scaffolding protein dimerization is an important step in procapsid assembly (Parker et al., 1997). (The role of the tetrameric form in assembly, if any, is not known.) One possibility is that the scaffolding proteins bind to coat protein monomers and induce a conformational change in the coat protein which renders it competent for assembly. Self-association of the scaffolding protein could also assist in the conformational switching of the coat subunits into their appropriate quasiequivalent conformations (Johnson, 1996; Johnson and Speir, 1997) and also may provide the driving force for pulling the coat protein subunits together and adding them to the growing procapsid.

At least 11 amino acids at the extreme carboxyl terminus of the P22 scaffolding protein are required for procapsid assembly and binding to the coat protein (Parker et al., 1998). A similar requirement for about 15±25 residues at the carboxyl terminus has been described for the scaffolding proteins from the related viruses herpes simplex type I (HSV-1; Matusick-Kumar et al., 1995; Hong et al., 1996) and cytomegalovirus (CMV; Beaudet-Miller et al., 1996; Wood et al., 1997). The scaffolding proteins from all three of these species have a pattern of hydrophobic residues at the carboxyl terminus that strongly suggests that they form amphipathic helices (Cohen and Parry, 1990), and helicity in this domain has been shown to be required for scaffolding/major capsid protein interactions in HSV-1 (Hong et al., 1996). For the P22 scaffolding protein, circular dichroism, Raman spectroscopy, and NMR have indicated that when 11 residues are removed from the carboxyl terminus, the protein loses a significant amount of helicity. Interestingly, approximately 20 additional residues also become disordered (Tuma et al., 1998a).

When the amino acid sequence of the carboxyl-terminal 30 residues of the P22 scaffolding protein is analyzed in terms of its pattern of polar and nonpolar amino acids, it becomes apparent that this region has a strong tendency to form a helix-loop-helix motif (Fig. 1). The stripe of hydrophobic residues on one face of the putative carboxyl-terminal helix (approximately residues 290±300) is positioned to bury against a similar stripe contributed by a second helix (approximately residues 275±285). Preliminary NMR data obtained from a truncated scaffolding protein support this model (Y. Sun, M.H.P., P. Weigele, P.E.P., S. Casjens, and N. Krishna, unpublished results). [We refer to this motif as a "helix-loop-helix" to avoid any confusion with the "helix-turn-helix" motifs commonly found in DNA- and calcium-binding proteins. It should be stressed, however, that the structural evidence available so far suggests that this loop is rather short (probably about five residues) and thus does not appear to protrude to a significant extent. Unlike the helix-turn-helix motifs found in many DNA- and calcium-binding proteins, this loop causes a nearly 180° change in the direction of the polypeptide chain. In this way, it appears to resemble the β-hairpin turns found in many β-sheet and four-helix-bundle proteins.]

One rather striking feature of this putative coat protein-binding domain is the number of basic residues that are found on the outer face of the carboxyl-terminal helix (Fig. 1). As many as six positively charged residues are clustered together on this face. This highly unusual arrangement led us to investigate the possibility that this domain interacts with the coat protein primarily through electrostatic interactions. We therefore tested the effects of high ionic strength on scaffolding/coat protein binding in the procapsid to determine whether this binding can be weakened by screening the postulated electrostatic
interactions. After the scaffolding protein is removed, a large number of negatively charged residues on the interior surface of the coat protein subunits might repel each other and destabilize the procapsid. Therefore we also investigated the possibility that the relief of this electrostatic repulsion constitutes the "trigger" that sets off the expansion of procapsids into the stable, mature capsid form.

RESULTS

High ionic strength inhibits procapsid assembly

Procapsid assembly reactions were carried out in vitro by adding the scaffolding protein to a solution of monomeric coat protein in a cuvette maintained at 20°C. Both proteins had been dialyzed into either buffer B (50 mM Tris HCl, 25 mM NaCl, 2 mM EDTA, pH 7.6) or buffer B containing 1000 mM NaCl. Procapsid assembly was monitored by recording the optical density at 250 nm due to light scattering (Prevelige et al., 1988). As depicted in Fig. 2, the presence of 1000 mM NaCl completely inhib-

FIG. 2. High ionic strength inhibits procapsid assembly in vitro. Monomeric coat protein was placed into a cuvette maintained at 20°C, and assembly was initiated by the addition of the wild-type scaffolding protein. Experiments were carried out in the presence of either 25 mM (circles) or 1000 mM (crosses) NaCl in buffer B. Procapsid assembly was monitored by recording the optical density at 250 nm due to light scattering. Data were recorded at 7.5-s intervals; only every third data point is shown for the 1000 mM NaCl incubations. The final concentrations of the coat and scaffolding proteins were 17 and 9.4 \( \mu M \), respectively.
come to equilibrium, and then isolated the products by centrifugation and analyzed their composition. The scaffolding protein was added to procapsid shells in the presence of either 25 or 1000 mM NaCl and allowed to equilibrate for 4 h at 20°C. To remove unbound scaffolding protein, the procapsids were pelleted by centrifugation through 10% sucrose solutions containing either 25 or 1000 mM NaCl in buffer. The pellets were analyzed by SDS–PAGE, as depicted in Fig. 3a. The presence of 1000 mM NaCl greatly reduced the amount of scaffolding protein which bound to the procapsids. Based on densitometry of the gels and the molecular masses of the scaffolding and coat proteins (Eppler et al., 1991), we calculated a total of 324 ± 25 scaffolding protein molecules per procapsid when binding was carried out in the presence of 25 mM NaCl, in reasonable agreement with the in vivo complement of approximately 250±300 per procapsid (Casjens and King, 1974; Eppler et al., 1991).

When binding was carried out in the presence of 1000 mM NaCl, however, only 94 ± 12 scaffolding protein molecules bound to each procapsid. This result suggests that increasing the NaCl concentration from 25 to 1000 mM reduced the average number of scaffolding protein subunits which bound to each procapsid by about threefold.

If nonspecific aggregates of the scaffolding protein are forming during these experiments, they might pellet along with the procapsids. Therefore we also analyzed the products of scaffolding protein/procapsid shell binding experiments by sedimenting the products through sucrose gradients.
5±20% sucrose gradients since this procedure separates particles with a higher degree of resolution. Figure 3b shows that the presence of 1000 mM NaCl reduced the scaffolding/procapsid binding when the particles were analyzed by sucrose gradient sedimentation. [The particles sediment more slowly in the presence of the higher salt concentration. Although empty procapsid shells do sediment slightly more slowly than full procapsids, the large difference in the rate of sedimentation observed here is likely due to an increase in the buoyant density and viscosity of the solution. In control experiments conducted with a nonbinding scaffolding protein fragment (residues 141–292; Parker et al., 1998) the shells displayed the same shift in position when the salt concentration was increased (data not shown).] The inhibition of scaffolding protein binding by 1000 mM NaCl appeared to be much greater in Fig. 3b than in Fig. 3a; the number of scaffolding molecules bound per procapsid decreased by a factor of about 10 in the presence of 1000 mM NaCl when the particles were isolated by sucrose gradient sedimentation. This discrepancy with the values calculated from Fig. 3a may be due to incomplete removal of the unbound scaffolding protein from the pellets which were analyzed in Fig. 3a. In any case, the results depicted in Figs. 3a and 3b suggest that 1000 mM NaCl lowers the average number of scaffolding protein molecules that can bind to a procapsid by at least threefold, suggesting that electrostatic interactions play a significant role in this binding.

When the scaffolding protein is added to procapsid shells, the binding can also be followed by measuring increases in the light scattering of the particles (Greene and King, 1994; Parker et al., 1998). Figure 3c shows the changes in light scattering observed when scaffolding protein was added to procapsid shells in the presence of increasing concentrations of salt. At 25 mM NaCl, the scattering increase displayed an initial burst which was essentially complete within 1±2 min. About half of this increase occurred within the mixing dead time (5±10 s). At 25 mM NaCl, a significant second phase of scattering increase occurred between 5 and 220 min. This is consistent with previous findings that the change in light scattering upon addition of the scaffolding protein to procapsid shells comprises at least two kinetic phases, with relaxation times on the order of 1 and 45 min, respectively (Greene and King, 1994; Parker et al., 1998).

As the NaCl concentration was increased from 25 to 1000 mM, the amplitude of the overall increase in light scattering progressively decreased, with no further effect being observed at 1750 mM NaCl. (For the SDS-PAGE analysis depicted in Figs. 3a and 3b, NaCl concentrations of 500 and 1750 mM were not employed.) The amplitude of the change in light scattering when the reactions were judged to be complete (240 min) was approximately threefold lower at 1000 mM than at 25 mM NaCl, suggesting that fewer scaffolding protein molecules were bound per procapsid and supporting the results depicted in Fig. 3a. When the ionic strength was increased, the slow kinetic phase observed at 25 mM NaCl disappeared. The faster phase decreased in amplitude as the ionic strength was increased, although the relaxation time of this phase did not appear to be affected.

To ascertain whether the decrease in binding caused by high ionic strength was due to irreversible conformational changes in the coat protein subunits, we incubated procapsid shells in buffer containing either 25 or 1000 mM NaCl for 2 h at room temperature and then dialyzed both samples into the low-salt buffer. Scaffolding protein binding experiments were carried out as depicted in Fig. 3a; no difference in the relaxation times or amplitudes of the light scattering increases were observed between the two samples (data not shown).

The data presented in Figs. 2 and 3 demonstrate that high ionic strength interferes with the binding of the scaffolding protein to the coat protein, suggesting that electrostatic interactions modulate this binding. A likely candidate for this interaction is that the positively charged amino acids at the carboxyl terminal of the scaffolding protein bind to complementary groups of negative charges on the coat protein. However, the scaffolding protein is capable of self-association, and in vitro assembly experiments conducted with a covalent dimeric mutant scaffolding protein (R74C/L177I) have suggested that dimerization is required for assembly (Parker et al., 1997). We therefore addressed the question of whether the salt-induced inhibition of scaffolding/coat protein binding is due to a decrease in the scaffolding protein’s ability to dimerize.

High ionic strength decreases scaffolding protein self-association; binding of covalent dimers is nevertheless inhibited by salt.

Sedimentation equilibrium experiments have demonstrated that the scaffolding protein forms a mixture of monomers, dimers, and tetramers in the presence of 25 mM NaCl (Parker et al., 1997). Other data suggest that the dimeric form is important for procapsid assembly. Since the self-association of the scaffolding protein is relatively weak, we thought it possible that high salt concentrations might interfere with dimer formation and that the observed inhibition of binding to coat protein might be caused by inhibition of scaffolding protein dimerization. We therefore carried out sedimentation equilibrium experiments in the presence of 25 and 1000 mM NaCl. Fitting of the data using two different computer programs which employ different fitting algorithms returned a value of 74±5 μM for the monomer/dimer dissociation constant at 25 mM NaCl and 20°C, in excellent agreement with the value of 78 μM determined previously (Parker et al., 1997; see also Materials and Meth-
In the presence of 1000 mM NaCl, however, the dimer dissociation constant was $170 \pm 6 \mu$M. Thus increasing the salt concentration reduced the free energy of dimerization by only about 7% (from 5.5 kcal/mol in 25 mM NaCl to 5.1 kcal/mol in 1000 mM NaCl). We were unable to obtain consistent values for the dimer/tetramer dissociation constants using the two different fitting algorithms for either salt concentration, a result which we interpret as indicating that the tetrameric form was not sufficiently populated to provide an accurate estimate of this value. However, the tetrameric form of the scaffolding protein does not appear to be required for assembly, since a truncated scaffolding protein which forms monomers and dimers, but not tetramers, can assemble procapsids (Parker et al., 1998; Tuma et al., 1998a).

Since increasing the NaCl concentration from 25 to 1000 mM caused a small but significant reduction in the degree of scaffolding protein dimerization, we tested the effect of high ionic strength on binding for a mutant scaffolding protein which cannot dissociate into monomers. A mutant scaffolding protein, R74C/L177I, contains a single cysteine residue per subunit and forms disulfide-crosslinked covalent dimers which are fully active in assembling procapsids (Greene and King, 1996; Parker et al., 1997). If the salt-induced inhibition of scaffolding/coat protein binding is solely due to blockage of dimer formation, then binding of the covalent dimeric mutant to the procapsid would be expected to be unaffected by high ionic strength. We tested this by performing re-entry experiments with the covalent dimeric scaffolding protein, using identical conditions to the experiments described above for the wild-type scaffolding protein. The data depicted in Figs. 4a and 4b suggest that binding of the covalently crosslinked dimeric scaffolding protein to procapsid shells is inhibited by 1000 mM NaCl. The number of R74C/L177I scaffolding protein subunits bound per procapsid (expressed in terms of the monomer) was calculated to be $202 \pm 12$ and $132 \pm 9$ in the presence of 25 and 1000 mM NaCl, respectively, when the particles were sedimented through 10% sucrose (Fig. 4a). This is only about a 1.5-fold decrease as opposed to the threefold decrease observed for the wild-type protein. When sedimented through sucrose gradients (Fig. 4b), the corresponding reduction in the number of scaffolding subunits bound was about 3.5-fold, in contrast to the 10-fold observed with the wild type. Therefore it appears that high ionic strength can still inhibit binding of scaffolding protein.
covalent dimers to the procapsid, although the degree of inhibition was less pronounced than for a scaffolding protein which can dissociate into monomers. This suggests that self-association of the scaffolding protein might contribute to the strength of the binding interaction between the scaffolding protein and the procapsid shell.

In Fig. 4c, the kinetics of R74C/L177I scaffolding protein binding are shown in the presence of 25 and 1000 mM NaCl. Interestingly, even at low ionic strength, the slow kinetic phase seen with the wild-type protein (relaxation time of about 45 min) was not observed. In the presence of 1000 mM NaCl, the amplitude of the fast phase was decreased, supporting the finding that high ionic strength inhibits the binding of the covalent dimeric scaffolding protein to the procapsid (Figs. 4a and 4b). Therefore the salt-induced inhibition of scaffolding protein binding to the coat protein appears to be caused primarily by direct inhibition of binding and not by inhibition of scaffolding protein dimerization.

One possible reason for the decreased binding of the scaffolding proteins in the presence of 1000 mM NaCl could be salt-induced conformational changes in the protein. We therefore measured the circular dichroism spectra of the wild-type scaffolding protein and the R74C/L177I mutant in the presence of 25 and 1000 mM NaCl. Both proteins had spectra which are characteristic of highly $\alpha$-helical proteins (data not shown), as previously observed (Teschke et al., 1993; Tuma et al., 1996). The higher salt concentration made no appreciable difference in the spectrum of the R74C/L177I covalent dimeric mutant, and only a slight (~5%) difference in the spectrum of the wild-type protein, which we attribute to the greater prevalence of monomers at the higher salt concentration. Therefore, it seems unlikely that the salt-induced inhibition is due to secondary effects of salt concentration on the scaffolding protein structure.

The covalent dimeric scaffolding protein was somewhat more resistant to the effects of high ionic strength than the wild-type protein. This suggests that scaffolding protein self-association within the procapsid stabilizes the binding to the coat protein. To test this, we measured the ability of 1000 mM NaCl to extract scaffolding proteins from procapsids. There was no significant difference in the amount of scaffolding protein remaining in the procapsid after 4 h compared to control experiments conducted in 25 mM NaCl (data not shown). Similar results have been observed using a variety of salts (B. Greene and J. King, personal communication). This suggests that self-association of the scaffolding protein within the tightly packed procapsid cooperatively stabilizes the binding to the coat protein. Since denaturants such as GuHCl can extract the scaffolding protein, we believe that partial denaturation of the coat protein-binding domain of the scaffolding protein is necessary for extraction (Tuma et al., 1996).

High ionic strength inhibits heat-induced expansion of procapsid shells

After the P22 procapsid is assembled, DNA is packaged into the procapsid, and the scaffolding protein exits. Once DNA packaging is complete, the procapsid expands approximately 10% in radius (Earnshaw et al., 1976) via an exothermic process (Galisteo and King, 1993). The procapsid can thus be viewed as a metastable structure which must overcome an activation energy barrier to attain its more stable mature capsid state. This barrier has been estimated to be approximately 35 kcal/mol of procapsid shells at 37°C (Tuma et al., 1998b). While the structure of the coat protein suggests that this expansion takes place via a hinge movement of two domains of the coat protein subunits (Casjens, 1979), the driving force for overcoming the activation energy barrier is not known.

We have provided evidence that suggests that electrostatic interactions between the scaffolding protein carboxyl-terminal domain and the coat protein subunits provide a substantial portion of the binding energy of scaffolding/coat protein interaction in the procapsid. Since the coat protein-binding domain of the scaffolding protein has a high density of positive charges, this implies that the complementary domain in the coat protein has an overall negative charge. If so, removal of the scaffolding proteins during DNA packaging might destabilize the procapsid shell by causing an increase in electrostatic repulsion among the negative charges on the coat protein subunits. Furthermore, the entry of DNA, a polyanion, might increase this instability even more. Thus it seems plausible that the coat protein subunits might shift position to move these negatively charged residues away from each other. This may provide a major portion of the driving force for expansion of the procapsid.

To test this, we investigated the effect of increasing the NaCl concentration from 25 to 1000 mM on the rate of procapsid expansion in vitro. Procapsid shells can be induced to expand by heating at 45±70°C (Galisteo and King, 1993; Tuma et al., 1998b), and the percentage of particles which have expanded at a given time can be quantified by native gel electrophoresis. In Fig. 5, the degree of procapsid expansion at 64°C in the presence of either 25 or 1000 mM NaCl is shown at various times. High ionic strength clearly inhibited the heat-induced expansion of the procapsids. Similar experiments were conducted at 55, 60, and 71°C. The percentages of shells which had expanded after 2 h at the indicated temperatures are listed in Table 1. For all temperatures tested, the presence of 1000 mM NaCl greatly inhibited expansion. The kinetics of expansion were also affected, in that expansion was slower in the presence of 1000 mM NaCl at all four temperatures (data not shown).
DISCUSSION

The construction of icosahedral viruses with T number > 1 requires that the coat protein subunits be able to adopt multiple quasiequivalent conformations within the lattice (Caspar and Klug, 1962). For most viruses with T number < 7, all of the information required for coat protein subunit conformational switching is contained within the subunits themselves. However, for viruses with T number ≥ 7, such as dsDNA phage (Casjens and Hendrix, 1988), herpesviruses (Hong et al., 1996; Wood et al., 1997), and adenoviruses (D'Halluin et al., 1978; Hasson et al., 1989, 1992) the assistance of a scaffolding protein, which both directs the form determination and accelerates the assembly process, is required. Therefore, understanding of the assembly processes for these viruses requires insight into the mechanisms by which scaffolding proteins interact with the coat or capsid proteins.

The assembly of the bacteriophage P22 has been characterized in considerable detail both in vivo and in vitro and serves as a model system to examine the structural and biochemical aspects of viral assembly (Prevelige and King, 1993). We report here that high salt concentrations completely inhibit the in vitro assembly of procapsids (Fig. 2) and partially inhibit interactions between the P22 scaffolding protein and the coat protein subunits in the procapsid (Figs. 3 and 4). We postulate that positively charged amino-acid residues in the scaffolding protein form a set of salt bridges with complementary regions of negative charge in the coat proteins. Many lines of spectroscopic evidence (Tuma et al., 1998a), along with standard methods of protein sequence analysis (Cohen and Parry, 1990; Eppler et al., 1991), suggest that the carboxyl-terminal domain of the scaffolding protein, which is required for coat protein binding (Parker et al., 1998), forms a helix-loop-helix motif. This motif would present six positive charges on one external face (Fig. 1), providing a somewhat unstable group of like charges which would be expected to seek neutralization by binding to the coat protein. (The P22 coat protein has an overall negative charge, both in the monomeric form and in the procapsid.) Raman spectroscopy has suggested that the helicity of the scaffolding protein increases upon binding to the coat proteins (Tuma et al., 1996); this may result from stabilization of the carboxyl-terminal helix-loop-helix domain upon binding.

We have previously demonstrated that the carboxyl-terminal domain of the scaffolding protein binds to coat protein monomers. As depicted in Fig. 6, this process could neutralize negatively charged residues in the coat protein subunit and promote conformational switching of the subunit into the assembly active form(s). Since scaffolding protein dimerization appears to be important for assembly (Parker et al., 1997), we believe that the energy of scaffolding dimerization can be used to bring coat protein subunits into the close proximity required for binding. Presumably, the required quasiequivalent conformational switching (Johnson, 1996; Johnson and Speir, 1997) of the coat protein occurs during this process, by a mechanism which remains unknown.

We are currently investigating the binding of the P22 scaffolding protein to procapsid shells using isothermal...
likely to be important in virus assembly. Since the scaffold protein carboxyl terminus recognizes complementary patches of negatively charged residues in two domains of the coat protein and induces a conformational change in the coat protein upon binding. The scaffolding proteins dimerize, pulling the bound coat protein subunits together and forming the correct coat protein conformations for assembly of the procapsid. After the procapsid is assembled and DNA packaging commences, the scaffolding proteins are removed, leaving a high density of negative charges in close proximity in the coat protein subunits. The application of heat to procapsids from which the scaffolding proteins have been extracted in vitro, or packaging of polyanionic DNA in vivo, could provide a driving force to induce the two coat protein domains to shift position relative to one another, moving the negative charges farther apart and relieving the electrostatic repulsion. This relief of electrostatic repulsion may provide the "trigger" which permits capsid expansion and maturation to occur once DNA packaging is complete.

Why would a virus use electrostatic interactions between its coat and scaffolding proteins? Many protein–protein complexes involve the binding of oppositely charged patches on the two proteins; examples include the complex between the leech protein hirudin and the blood-clotting enzyme thrombin (Betz et al., 1991) and the interaction between the enzyme barnase and its cellular inhibitor barstar (Schreiber and Fersht, 1996). Estimates of the increase in binding free energy contributed by electrostatic attractions vary widely but generally fall within the range of 0.5–5 kcal/mol per pair of residues (Stone et al., 1989; Betz et al., 1991; Horton and Lewis, 1992; Nakamura, 1996), with the strength of the interaction decreasing with distance (Loewenthal et al., 1993). However, isolated salt bridges can sometimes destabilize protein–protein interactions, since the formation of an ion pair involves a large desolvation energy penalty which may not be fully compensated by the electrostatic attraction (Hendsch and Tidor, 1994; Froloff et al., 1997; Chong et al., 1998). Because salt bridges can either stabilize or destabilize protein–protein interactions, it has been suggested that electrostatic interactions are useful mainly for conferring specificity, while the major contribution to stability is made by hydrophobic interactions (Hendsch and Tidor, 1994). Electrostatic attractions, being active at relatively long distances, can also increase the association rate by "steering" the two proteins together (Schreiber and Fersht, 1996; Gabdoulline and Wade, 1998; Vijayakumar et al., 1998), a process which is likely to be important in virus assembly. Since the scaffolding protein must be released from the procapsid during maturation, it should not bind too tightly. Electrostatic interactions, being of intermediate strength, might provide just the right degree of binding affinity to allow the scaffolding protein to be released.

In P22, as in other dsDNA phage (King and Chiu, 1997), DNA is packaged by a headful mechanism in an ATP-dependent fashion. During packaging the scaffolding protein is removed and the coat protein shell expands and becomes more angular in appearance. In P22, the scaffolding protein is believed to escape via holes in the centers of the hexameric clusters of coat protein subunits (Prasad et al., 1993); these holes become closed during the expansion process. Raman spectroscopy has indicated that there is little change in the secondary structure of the P22 coat protein during capsid expansion (Prevelige et al., 1993), supporting the idea first proposed by Casjens (1979) that capsid expansion involves the movement of two coat protein domains via a "hinge-bending" mechanism. The hinge region between the two domains has been mapped, by limited proteolysis of the 430-amino-acid coat protein, to approximately residues 175–205 (J. Lanman and P.E.P., unpublished results), indicating that the two domains are of similar size.

While the mature, expanded form of the capsid is more thermodynamically stable than the procapsid (Galisteo and King, 1993), procapsids and empty procapsid shells are nevertheless quite stable below about 40°C. Therefore a driving force must be applied to overcome the activation energy barrier to expansion. Given our findings that the scaffolding/coat protein interactions have a large electrostatic component (Figs. 2±4), it is reasonable to assume that the coat protein subunits have a considerable number of negatively charged amino-acid residues in close proximity on the inner surface of the procapsid. It is possible that the exit of the scaffolding protein destabilizes the cap-
sid by increasing the electrostatic repulsion between negatively charged domains of the coat protein subunits. As DNA, a polyanion, is packaged into the procapsid, the density of negative charges may be increased to such an extent as to provide the driving force necessary for expansion. Shifting of the coat protein domains may provide a way of reducing this repulsion, with the result that the procapsid expands into its mature form (Fig. 6b). The finding that high ionic strength inhibits heat-induced procapsid expansion (Fig. 5, Table 1) supports this model. Presumably, the presence of high salt concentrations screens the electrostatic repulsion and stabilizes the procapsid shell.

The relief of electrostatic repulsion in the coat proteins may be a mechanism for triggering the expansion of other dsDNA phage and possibly for other types of viruses as well. Removal of Ca$^{2+}$ by chelation triggers expansion in tomato bushy stunt virus by causing an electrostatic repulsion between aspartic acid residues on adjacent coat protein subunits (Robinson and Harrison, 1982). Dialyzing T4 phage into buffer of very low ionic strength leads to spontaneous expansion of the procapsids (Carascosa, 1978). In general, 10±30% of the genome must enter the procapsids of dsDNA phages before expansion occurs (Earnshaw and Casjens, 1980; Bjornsti et al., 1983; Hohn, 1983; Shibata et al., 1987), suggesting that the introduction of DNA is part of the signal for triggering expansion. Rao and Black (1985) have demonstrated that both expanded T4 capsids and mutants that cannot expand can package DNA in vitro, suggesting that expansion does not provide the driving force for DNA packaging.

King and Chiu (1997) have also proposed that DNA packaging triggers expansion in dsDNA phage. In their model, the scaffolding protein masks DNA-binding domains on the inner surface of the coat protein shell (which presumably would be positively charged), and the removal of the scaffolding protein allows DNA to bind to these surfaces. Expansion of the shell would bury the DNA-binding domains, facilitating release of the DNA upon infection of the host. Our data suggest that the scaffolding-binding domains on the inner surface of the P22 procapsid have an overall negative charge. However, this does not rule out the possibility that positively charged DNA-binding surfaces exist at other points on the inner surface of the procapsid. It should be noted that while these experiments suggest that scaffolding/coat protein binding is mediated by electrostatic interactions, they do not address the potential role of electrostatic interactions during the formation of coat/coat contacts.

We have presented a model for the binding of scaffolding proteins within the P22 procapsid, and for the subsequent maturation of the capsid, which involves electrostatic interactions as a driving force. Our findings with respect to P22 may be applicable to other dsDNA phage as well, although it should be noted that, while P22 and T4 have the potential to form amphipathic helices near their carboxyl termini, T3, T7, and λ do not (Tuma et al., 1998a). Eppler et al. (1991) have noted that many phage scaffolding proteins are highly charged and that the P22, T7, and T4 scaffolding proteins all have highly asymmetric charge distributions (with positively charged residues concentrated near the carboxyl terminal).

Whether this model is applicable to other viruses remains to be seen. Although herpesviruses such as HSV-1 and CMV have many similarities to P22, the electrostatic model we have presented may not apply to them. While the scaffolding proteins of both HSV-1 and CMV appear to have short amphipathic helical domains at their carboxyl termini which are required for activity (Beaudet-Miller et al., 1996; Hong et al., 1996), there is no evidence that they form helix-loop-helix motifs. Furthermore, neither of these proteins has the high concentration of charged residues in the carboxyl-terminal region which is found in the P22 scaffolding protein. Hong et al. (1996) proposed that the HSV-1 scaffolding protein binds to the major capsid protein via a hydrophobic interaction. Both HSV-1 and CMV express proteases which cleave the scaffolding proteins near their carboxyl termini, a process which is required for release of the scaffolding proteins. This suggests that the scaffolding proteins in these viruses bind more tightly, perhaps by hydrophobic interactions, and thus require proteolysis to be released from the procapsid. Like P22, the HSV-1 procapsid expands during maturation (Trus et al., 1996). Whether electrostatic repulsion provides the driving force for expansion of HSV-1 is unknown.

MATERIALS AND METHODS

Preparation of proteins

P22 procapsids were obtained as described previously (Prevelige et al., 1988). Briefly, phage containing amber mutations in DNA packaging genes were used to infect Salmonella typhimurium. After lysis of the bacteria, procapsids were harvested by centrifugation and purified by size-exclusion chromatography. Empty procapsid "shells" were obtained by repeated extraction with 0.5 M guanidinium hydrochloride (GuHCl), followed by sedimentation through a solution of 10% (w/w) sucrose in buffer "B" (50 mM Tris HCl, 25 mM NaCl, 2 mM EDTA, pH 7.6). SDS-PAGE indicated that the resulting shells were >99% pure coat protein. The preparations contained <1% expanded shells, as indicated by electrophoresis in agarose gels (Galisteo and King, 1993; see below). Procapsids and empty shells were stored at 4°C.

The GuHCl extracts were used to isolate the wild-type scaffolding protein by ion-exchange chromatography (Prevelige et al., 1988; Parker et al., 1997). The R74C/L177I mutant scaffolding protein was purified from procapsids using a slightly different procedure (Parker et al., 1997). SDS-PAGE indicated that all scaffolding proteins were >99% pure and that the R74C/L177I covalent dimeric mutant contained >99% covalently crosslinked...
dimeric protein. The scaffolding proteins were stored at −20°C in buffer B, and all experiments were conducted using this buffer unless otherwise noted.

Procapsid assembly reactions

Monomeric coat protein was prepared by dissociating procapsid shells with several volumes (about 2 ml) of 6 M GuHCl in buffer B, followed by dialysis against 2 × 500 ml of buffer B (Prevelige et al., 1988; Parker et al., 1997, 1998). For experiments conducted at high ionic strength, buffers. Aliquots of 400 μl of the protein was dialyzed against the same low- and high-salt concentrations of 14 (1 M). A scaffolding mutant was added to a solution of procapsid scaffolding protein re-entry kinetics

Wild-type scaffolding protein or the R74C/L177I covalent dimeric mutant was added to a solution of procapsid shells (0.6 mg/ml final concentration) in a cuvette maintained at 20°C, and assembly was initiated by the addition of 50 μl of the scaffolding protein. Procapsid assembly was monitored by following the increase in optical density at 250 nm due to light scattering. Readings were taken at 7.5-s intervals using a 0.5-s read-averaging time. The final concentrations of the coat and scaffolding proteins were 0.81 mg/ml (17 μM) and 0.32 mg/ml (9.4 μM), respectively.

Scaffolding protein re-entry kinetics

Wild-type scaffolding protein or the R74C/L177I covalent dimeric mutant was added to a solution of procapsid shells (0.6 mg/ml final concentration) in a cuvette maintained at 20°C. The scaffolding proteins were present at concentrations of 141 μM monomeric subunits, i.e., a 11-fold molar excess over the coat protein subunits in the shells. Experiments were carried out at 25 and 1000 mM NaCl in buffer B with the R74C/L177I mutant scaffolding protein, and at 25, 500, 1000, and 1750 mM NaCl with the wild-type scaffolding protein. Re-entry and binding were monitored by following the increase in optical density at 250 nm due to light scattering. Data points were obtained at 1-s intervals for the first 20 min (01-s read-average time), followed by a further 220 min at 45-s intervals (2-s read-average time). The magnitudes of the changes in light scattering caused by the binding of scaffolding proteins to procapsid shells were calculated by measuring the initial optical density of the procapsid shells before the addition of scaffolding protein, correcting for dilution, and then adding the optical density at 250 nm calculated for the scaffolding protein at that concentration. These values were then subtracted from the raw optical density values.

Composition of particles after scaffolding protein re-entry

Scaffolding proteins were mixed with procapsid shells and allowed to react for 4 h in the presence of either 25 or 1000 mM NaCl as described in the previous section. The 450-μl solutions were then layered onto 1 ml solutions of 10% (w/w) sucrose in buffer B containing either 25 or 1000 mM NaCl, and the procapsids were pelleted by centrifugation for 1 h at 60,000 revs/min, 20°C in a Beckman TLA-100.3 rotor. The supernatant was carefully decanted, and the pellets were dissolved in 100 μl of SDS-PAGE gel loading dye solution. Aliquots of 1 μl were analyzed by SDS-PAGE on 13% polyacrylamide gels followed by staining with Coomassie Blue R250 and scanning with an Alpha Imager 2000 densitometer (Alpha Innotech Corp., Hayward, CA).

In separate experiments, 200 μl of each reaction mixture was layered onto 5-ml linear gradients of 5±20% (w/w) sucrose in buffer B containing either 25 or 1000 mM NaCl atop 150-μl cushions of 60% CsCl in 20% sucrose, and centrifuged at 20°C for 35 min at 35,000 revs/min in a Beckman Sw55-Ti swinging-bucket rotor. The gradients were separated into 14 fractions and analyzed by SDS-PAGE as described above. Control experiments were carried out using a truncated scaffolding protein consisting of residues 141±292. This protein does not assemble procapsids or bind to the coat protein (Parker et al., 1998). None of the 141±292 scaffolding protein fragment was found in the fractions which contained procapsid shells at either salt concentration.

Analytical ultracentrifugation

Scaffolding protein was dialyzed against buffer B containing either 25 or 1000 mM NaCl. The protein was then centrifuged for 2 h at 60,000 revs/min in a Beckman TLA-100.3 rotor at 4°C to remove aggregates of s value greater than 20 which may have formed upon storage. Solutions of 0.5, 1.0, and 1.5 mg/ml were sedimented to apparent equilibrium (approximately 18±24 h) at 20°C at 15,000 and 22,000 rpm in a Beckman Optima XL-A centrifuge equipped with an eight-cell rotor. The absorbance at 277 nm was scanned versus the radial position for each cell, and the data were subsequently fit using Origin (MicroCal, Northampton, MA) and Multifit (L. Hollday, available on the World Wide Web at http://bio09.uthsc.edu/auc/xla2.html). Several models for self-association were tested and evaluated as described previously (Parker et al., 1997; Tuma et al., 1998a). Data were also obtained at 360 nm to detect any material which aggregated in the cells; data from these radial positions were discarded.

Various values for the extinction coefficient of the scaffolding protein at 280 nm have been reported, including 0.45 (Fuller and King, 1981), 0.48 (Teschke et al., 1993), and 0.57 ml-mg⁻¹-cm⁻¹ (Galisteo and King, 1993). In a previous publication in which the association constants for the scaffolding protein were determined by analytical ultracentrifugation (Parker et al., 1997), the value of 0.45 was employed. We have since recalculated the 280 nm extinction coefficient using the method of Gill and von Hippel (1989) and obtained a value of 0.53 ± 0.01 ml-mg⁻¹-cm⁻¹, in good
agreement with the value of 0.52 predicted by the method of Pace et al. (1995). The extinction coefficient at 277 nm for the 12 cm cell used in the analytical ultracentrifuge was calculated to be 0.65 ml·mg⁻¹ using Gill and von Hippel’s method; this value was used in this paper. (Sodium chloride at 1 M had no effect on the extinction coefficient.) The dimer dissociation constant of 91 µM reported previously (Parker et al., 1997) was recalculated to be 78 µM using the revised extinction coefficient.

Heat-induced expansion of procapsid shells

Shells at 0.9 mg/ml were dialyzed into buffer “C” (50 mM potassium phosphate, 25 mM NaCl, 2 mM EDTA, pH 7.6). Aliquots of 10 µl were placed into chilled thin-walled 500 µl PCR tubes. Ten microliters of either buffer C or buffer C containing 2000 mM NaCl was added, and the mixtures were overlaid with 20 µl of mineral oil. Samples were removed from ice and placed into a water bath at the indicated temperature. After the indicated time, each sample was removed from the bath and immediately placed into ice. (Separate samples were used for each time point.) Aliquots of each sample were diluted 10-fold in sample loading buffer (buffer C containing 30% v/v glycerol and 0.01% bromphenol blue); 20 µl aliquots were loaded onto gels of 1.2% agarose in buffer C. Samples were electrophoresed toward the anode for 7 h at 17 V/cm, 4°C. The gels were stained with Coomassie Blue for 1 h, destained for several days, and then scanned as described above. The percentage of shells which had expanded at each temperature and time was calculated by measuring the staining intensities of the expanded and unexpanded shells and expressed as a percentage of the total intensity. No dissociated coat protein was detected in the gels.

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