REGULATION OF COAT PROTEIN POLYMERIZATION BY THE SCAFFOLDING PROTEIN OF BACTERIOPHAGE P22

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ABSTRACT In the morphogenesis of double stranded DNA phages, a precursor protein shell empty of DNA is first assembled and then filled with DNA. The assembly of the correctly dimensioned precursor shell (procapsid) of *Salmonella* bacteriophage P22 requires the interaction of some 420 coat protein subunits with ~200 scaffolding protein subunits to form a double shelled particle with the scaffolding protein on the inside. In the course of DNA packaging, all of the scaffolding protein subunits exit from the procapsid and participate in further rounds of procapsid assembly (King and Casjens. 1974. *Nature (Lond.).* **251**:112–119). To study the mechanism of shell assembly we have purified the coat and scaffolding protein subunits by selective dissociation of isolated procapsids. Both proteins can be obtained as soluble subunits in Tris buffer at near neutral pH. The coat protein sedimented in sucrose gradients as a roughly spherical monomer, while the scaffolding protein sedimented as if it were an elongated monomer. When the two proteins were mixed together in 1.5 M guanidine hydrochloride and dialyzed back to buffer at room temperature, procapsids formed which were very similar in morphology, sedimentation behavior, and protein composition to procapsids formed in vivo. Incubation of either protein alone under the same conditions did not yield any large structures. We interpret these results to mean that the assembly of the shell involves a switching of both proteins from their nonaggregating to their aggregating forms through their mutual interaction. The results are discussed in terms of the general problem of self-regulated assembly and the control of protein polymerization in morphogenesis.

INTRODUCTION

If the structural proteins of microtubules, muscle fibers, ribosomes, viruses, or other organelles simply aggregated spontaneously as they were released from their ribosomes, a variety of complex structures would certainly assemble, but it is unlikely that they would be very closely related to the structures needed for biological function. For example, the subunits of bacterial flagella must not polymerize within the cytoplasm but only on the growing tip of pre-existing flagella. In the assembly of the tail of bacteriophage T4, the baseplate specifically initiates polymerization of the tail tube subunits. In the absence of the baseplate the tail tube subunits remain as soluble subunits (Kikuchi and King, 1975; Wagenknecht and Bloomfield, 1978).

Such regulation of the polymerization of protein subunits requires the existence of states of the protein which do not spontaneously polymerize. Fibrinogen is a familiar example of a protein in such a nonpolymerizing state: proteolytic processing is required for transformation to the polymerizing subunit.

However, the existence of different stable states does not necessarily depend on covalent cleavage. The analysis of the assembly pathways of bacterial viruses (Wood and King, 1979), bacterial flagella (Uratani and Asakura, 1972), and tobacco mosaic virus (Butler, 1978;

Schuster et al., this volume) reveals quite clearly that identical protein subunits can have different, stable states which do not spontaneously interconvert. Thus, the subunits of bacterial flagellae do not spontaneously polymerize in vitro; they must be bound by the growing tip of seed fragments from organized flagella. This surface then catalyzes a conformational change, which converts the bound subunit to an active site for the next step in the pathway (Asakura, 1970; Uratani et al., 1972). Similarly, the numerous species of subunits of the tail of bacteriophage T4 do not react with each other as free subunits in solution but only with the growing structure. Each subunit must be activated by incorporation into a specific intermediate complex. This then generates the active complex for binding the next subunit in the pathway (Kikuchi and King, 1975; King, 1980). Thus, a unique, sequential, stepwise assembly pathway ensues. Though these processes are still consistent with the self-assembly concept, they exhibit additional features and are more properly termed self-controlled or self-regulated assembly (Caspar, 1976; King, 1980).

Assembly of Closed Shells

In this paper we analyze the regulation of protein polymerization in the formation of shell structures. Though the geometry of the packing of subunits into icosahedral lattices is well understood (Caspar and Klug, 1962), the pathway of subunit interaction has turned out to be substantially more complex than originally imagined. The capsids of all the well-studied double-stranded DNA phages are assembled first as double protein shells empty of DNA. Once completed, the precursor shells play an active role in the encapsulation of the phage DNA (Casjens and King, 1975; Showe and Kellenberger, 1976; Murialdo and Becker, 1978). In all of these phages, substantial reorganization of the precursor shells occurs during the transition from the precursor to the mature capsid. Thus, the mature shell is not the direct product of the subunit polymerization process, and subunits derived from the mature shell are not in the state which has the property of assembly into shells. This is of course clearest in those cases, such as T4, where there are major proteolytic cleavages in the transition from the precursor shell to the mature shell (Laemmli, 1970; Showe and Kellenberger, 1976).

A feature of the assembly pathway of all the double stranded DNA phages that have been analyzed is the requirement for a major auxilliary protein, in addition to the major capsid protein itself. These assembly core or scaffolding proteins (Showe and Black, 1973; King et al., 1973; Casjens and King, 1974) share a number of general properties: (*a*) They are incorporated into the precursor shell; (*b*) they are absent from the capsid of the mature virus; (*c*) if removed from the infected cells (by mutation), the major coat subunits do not polymerize correctly; (*d*) The scaffolding proteins are removed from the precursor shell before or in concert with the DNA packaging process.

Fig. 1 shows the detailed morphogenetic pathway of one of the well studied dsDNA phages, P22, of *Salmonella typhimurium*. The 55,000-dalton major coat protein is the product of P22 gene 5 (gp5), while the 42,000-dalton scaffolding protein is the product of gene 8 (gp8). These proteins polymerize together with four minor protein species into the procapsid shell. The packaging of the DNA into this shell requires two additional proteins, the products of genes 2 and 3, which are not structural proteins. Rather they catalyze the transport of the DNA molecule into the precursor shell (Murialdo and Becker, 1978; Poteete and Botstein, 1979). If cells are infected with mutants defective in the genes for these proteins, procapsids accumulate inside the cell. These structures are stable and can be isolated with relative ease (Fig. 2). They sediment at ~240 S (King et al., 1973). In thin sections of infected cells, the procapsids appear to be double shelled structures (Lenk et al., 1975). Low angle x-ray

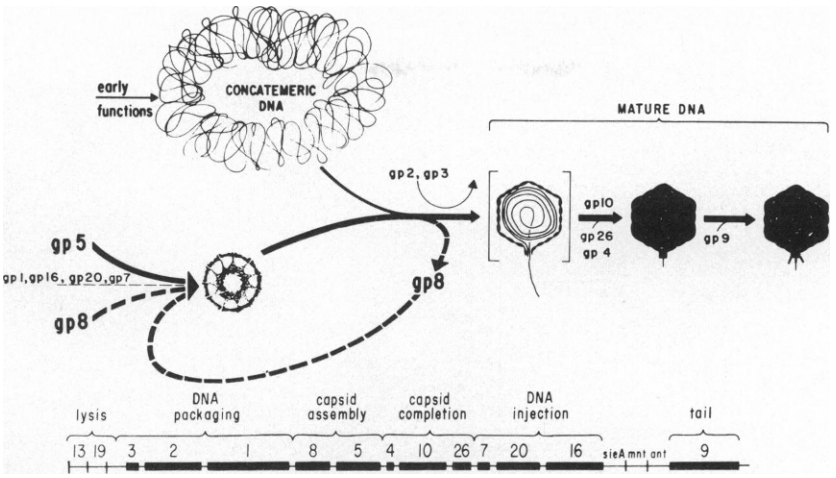

Figure 1 Pathway for the assembly of bacteriophage P22 and genetic map of the morphogenetic genes. This figure shows the organized structural intermediates and genetically controlled steps in the morphogenesis of bacteriophage P22. Proteins are named for the genes which specify them: thus gp5 is the product of gene 5. The first shell structure on the left is the procapsid, which is the precursor structure in the DNA packaging reaction. During the DNA packaging reaction scaffolding protein molecules (gp8) exit from the procapsid and are reused in subsequent rounds of procapsid assembly (King and Casjens, 1974). The newly filled capsid is shown in parentheses. This structure is unstable and loses its DNA, yielding an empty capsid. The action of the proteins specified by genes 4, 10 and 26 normally stabilizes the newly formed capsid and forms the site for tail spike attachment, the terminal step in phage maturation. The gp2 and gp3 proteins do not become part of the virus but are needed for DNA encapsulation (Botstein et al., 1973; Poteete and Botstein, 1979). The location of the genes controlling morphogenesis on the P22 chromosome is shown beneath the assembly pathway (Susskind and Botstein, 1978). The coat and scaffolding proteins are coded for by adjacent genes in a variety of dsDNA phages-T4, Lambda, T3, T7, and $\phi/29$ (Wood and King, 1979).

scattering analysis has established that they consist of a thick inner protein shell within a thinner outer shell of coat protein (Earnshaw et al., 1975). This general structure is shown in Fig. 3 *a*.

During a wild type infection, proheads lose their scaffolding protein core and expand to the radius of the mature capsid during DNA packaging. We have used purified P22 proheads as a source of coat protein which has not gone through this maturation step. By artificially inducing the release of the scaffolding protein core in vitro, we developed a simple procedure for the purification of both the coat and the scaffolding protein from P22 proheads. Using these preparations we have tried to determine if the scaffolding subunits self-assemble into an inner shell, followed by assembly of the outer shell, or whether both species copolymerize into a double shell.

MATERIALS AND METHODS

Phage and Bacterial Strains

All phage and bacterial strains were from the collections of D. Botstein or J. King and have been described previously (Botstein et al., 1973; Poteete and King, 1977; Susskind and Botstein, 1978). Proheads were isolated from suppressor strains of *S. typhimurium* infected with P22 phage carrying amber mutations in genes necessary for DNA packaging. The proheads accumulate as a result of the block to DNA packaging (Botstein et al., 1973; King et al., 1978).

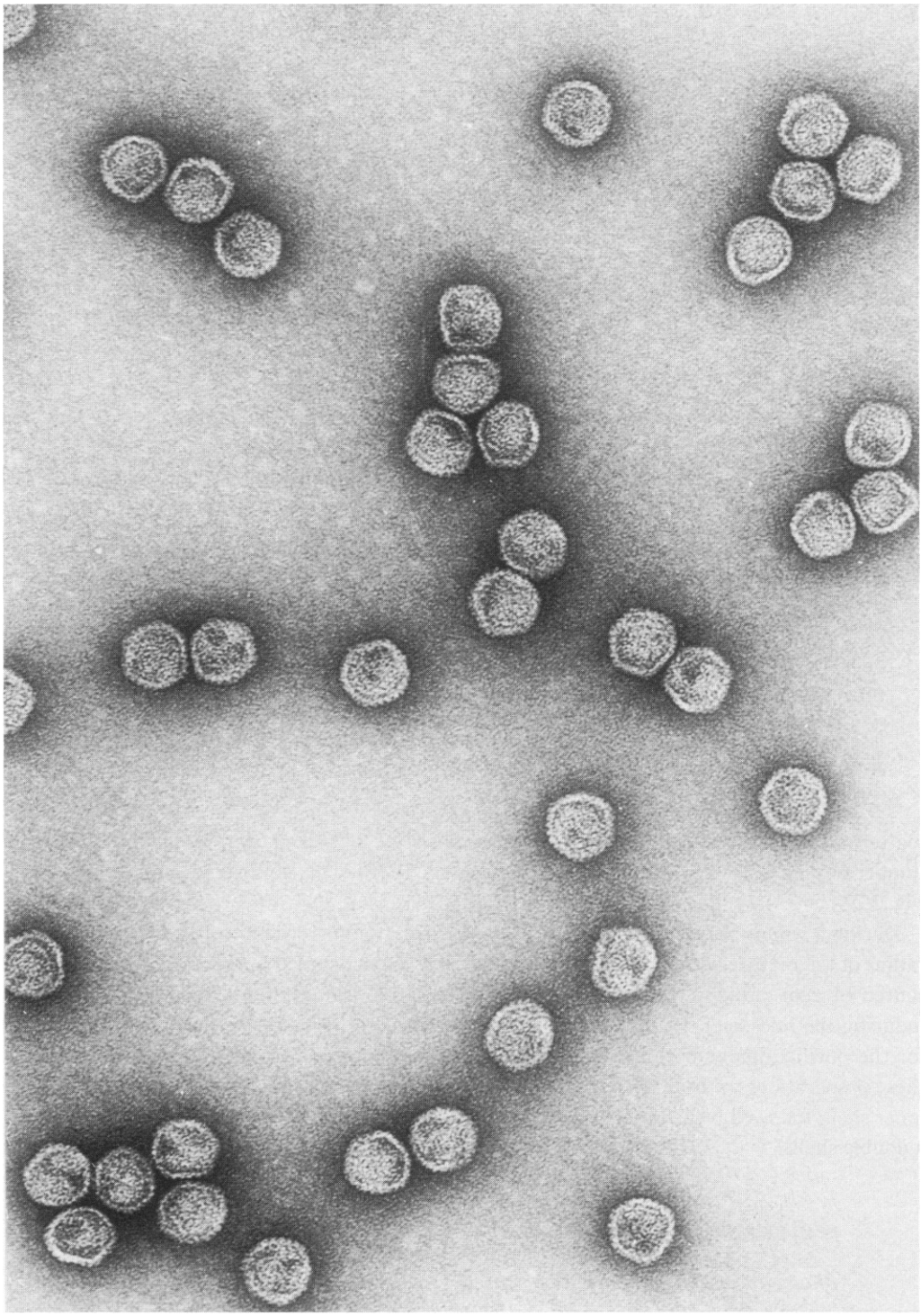

Figure 2 Electron micrograph of P22 procapsids. These particles have been isolated from cells infected with mutants defective in DNA packaging (Botstein et. al., 1973). The particles have been contrasted by negative staining with uranyl acetate. A thin ring of increased intensity of staining within the outer shell setting it off from the central stain excluding area can be observed. This corresponds to the region of low protein density between the coat and scaffolding subunits revealed by low angle x-ray scattering.

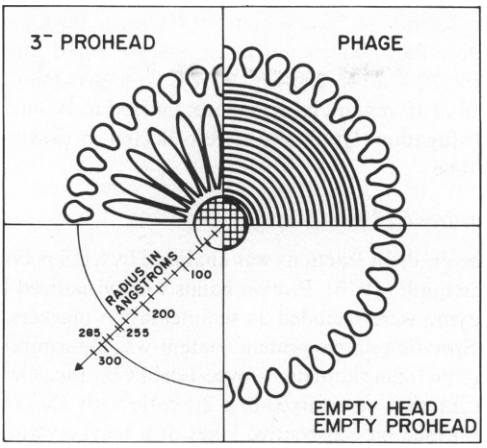

Figure 3 Protein shell organization in phage P22. The left hand panel shows a model of procapsid organization which fits both low angle x-ray scattering data (Earnshaw et al., 1976) and electron microscope observations (Casjens and King, 1974; Lenk et al., 1975). The coat protein shell of the mature phage is expanded with respect to the prohead dimensions. The DNA within the shell is concentrically coiled as shown (Earnshaw and Harrison, 1977; Earnshaw et al., 1978) but there are only 5–6 concentric coils. Empty shells illustrated in the lower right panel were prepared by extraction of the scaffolding protein from procapsids by treatment with SDS in vitro. Empty heads represent particles initially filled with DNA which have lost DNA during isolation. Fig. 1 reprinted from Earnshaw et al. (1976).

Media Chemicals

Buffer B consists of: 50 mM Tris-HCl, PH 7.6, 25 mM NaCl, 2 mM EDTA, 3 mM 2-β mercaptoethanol, and 1% glycerol. Ultrapure guanidine hydrochloride (GuHCl) was obtained from Schwartz-Mann, Div. Becton, Dickson & Co., Orangeburg, N.Y.. Guanidine hydrochloride solutions were made in buffer B. Bovine hemoglobin was purchased from Sigma Chemical Co., St. Louis, Mo., and chicken egg white lysozyme was purchased from Calbiochem Behring Corp., American Hoechst Corp., San Diego, Calif.. Sucrose solutions were made wt/wt in buffer B without glycerol.

Proteins

P22 coat and scaffolding protein were purified by selective disruption of purified P22 proheads with GuHCl, followed by gel filtration in GuHCl on Biogel A-5m and ion exchange chromatography on DEAE cellulose as described by Fuller (1979). Coat protein was freshly prepared from 2^- proheads and kept at 4°C in 2 M GuHCl. Scaffolding protein was purified by ion exchange chromatography on DEAE cellulose after release from 3^- 20^- proheads, and stored frozen in liquid nitrogen. After an aliquot of scaffolding protein was thawed, it was kept at 4°C.

In Vitro Assembly

To perform an assembly experiment, 300 μl of coat protein in 2 M GuHCl in buffer B was mixed with 100 μl soluble scaffolding protein in buffer B containing 50–70 mM extra NaCl (a result of the ion exchange purification step). The concentration of GuHCl in all reaction mixtures was 1.5 M GuHCl. The mixtures were dialyzed at 23°C or at 4°C against 100–500 ml of buffer B in two changes. Dialysis lasted for 12–18 h. Mixtures missing one of the proteins were made with the respective buffer. The ratio of coat to scaffolding protein varied from experiment to experiment. Most often the assembly reaction mixture contained scaffolding protein in slight excess over the coat protein as determined by Coomassie blue stain of SDS gel.

Sucrose Gradients

In vitro assembly mixtures were analyzed for assembly after dialysis by centrifugation on 5 ml, 5–20% sucrose (wt/wt) gradients with a 0.3-ml 60% sucrose (wt/wt) cushion. To display prohead sized structures two thirds of the way down the centrifuge tube, gradients were centrifuged for 40 min at

35,000 rpm at 20°C, or for 70 min at 35,000 rpm at 4°C in a Beckman SW50.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). To analyze small molecular weight components, gradients were centrifuged for 20 h at 45,000 rpm at 4°C in a Beckman SW50.1 rotor. 10 μl of 10 mg/ml hemoglobin and 10 ml of 10 mg/ml lysozyme were added to 100 ml of sample as sedimentation markers for the longer centrifugation. Gradients were collected in constant volume fractions from a pinhole at the bottom of the tube.

SDS Polyacrylamide Gel Electrophoresis

Protein composition of sucrose gradient fractions was analyzed by SDS polyacrylamide gel electrophoresis in the buffer system of Laemmli (1970). Protein bands were visualized by Coomassie blue staining. When hemoglobin and lysozyme were included as sedimentation markers, gels were cast with 12.5% instead of 10% acrylamide. Specific relative protein content was determined for each gradient fraction with a Joyce-Loebl recording microdensitometer (Joyce-Loebl Co., Inc., Gateshead-on-Tyne, England). Samples from each gradient fraction were mixed in a 2:1 ratio with 3X sample buffer, incubated for 2 min at 100°C, and electrophoresed on consecutive lanes of a polyacrylamide slab gel. After staining, lanes were scanned with the microdensitometer. The area under each peak was measured with a Neumonics integrator to determine the relative amounts of each polypeptide. The amount of a specific protein in a given gradient fraction was then expressed as a percent of the total amount of that protein recovered from the gradient.

Electron Microscopy

Samples of assembly reaction mixtures, or of sucrose gradient fractions, were applied to carbon coated, copper electron microscope grids and negatively stained by washing with 2% uranyl acetate, pH 4.5. Residual liquid was blotted off and the grids allowed to air dry before examination in a JEM 100B electron microscope.

RESULTS

Isolation of Soluble Scaffolding Subunits

To study the mechanism of assembly of procapsids from coat and scaffolding protein, we needed preparations of soluble subunits in relatively pure form. As starting material for purification of the proteins we used procapsids themselves, as in the experiments of Van Driel and Couture (1978) on T4 prohead assembly. Since the scaffolding protein which exits from P22 proheads during DNA packaging in vivo is biologically active in further rounds of procapsid assembly, it seemed reasonable to attempt to obtain active scaffolding protein subunits by inducing release of the protein from procapsids in vitro. Procapsids were first purified from cells infected with mutants of genes 2 or 3, which are blocked in DNA packaging (see Fig. 1). The purification was based on differential centrifugation and column chromatography[1] (King and Casjens, 1974; Earnshaw et al., 1976). The resulting procapsid preparations contained primarily coat protein, scaffolding protein, and the minor procapsid proteins gpl, gp7, gp16, and gp20.

Treatment of procapsids with appropriate concentrations of SDS, urea, or guanidine hydrocholoride resulted in the release of the scaffolding protein and the minor proteins, leaving the shell of coat protein intact[1] (Casjens and King, 1974). We suspect that the scaffolding protein molecules exit through the coat protein lattice. The resulting empty procapsids look intact in the electron microscope, and show no obvious orifice. However, it is difficult to rule out exit of the internal proteins through a single hole, or through breaks in the capsid. The ability to release the scaffolding protein from within intact proheads provides a

[1]Fuller, M., and J. King. Manuscript submitted for publication.

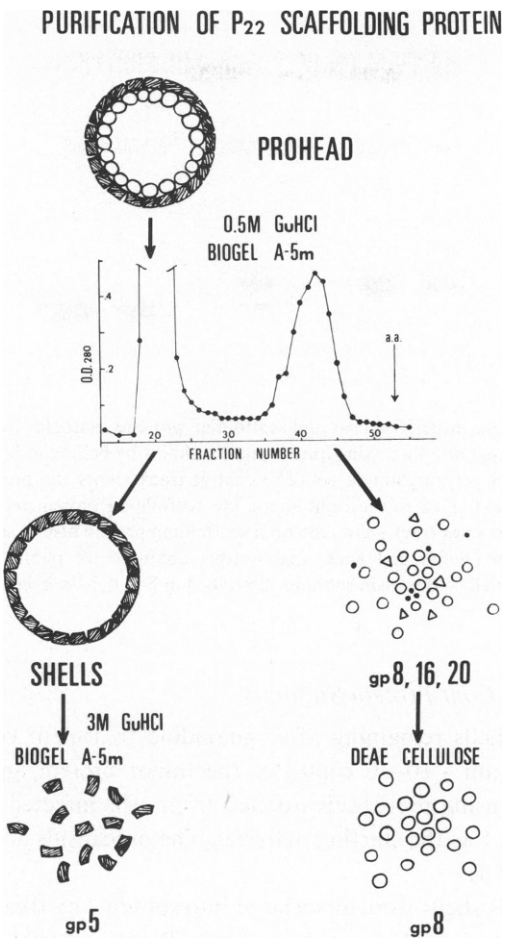

Figure 4 Scheme for the purification of coat and scaffolding subunits from isolated procapsids. The scaffolding protein was released from procapsids by treatment with 0.5 M guanidine gydrochloride. Free subunits were separated from intact shells of coat protein by gel filtration through a Biogel A-5m column. The scaffolding subunits were included in the column while the intact shells were found in the void volume. These shells were then dissociated into subunits with a higher concentration of guanidine hydrochloride. The coat protein subunits were separated from any remaining high molecular weight aggregates by an additional passage through a gel filtration column. These procedures are described in detail elsewhere.[1]

convenient means for obtaining both coat and scaffolding proteins from a population of procapsids as shown in Fig. 4.

For purification of the scaffolding protein, the procapsids were treated with 0.5 M guanidine hydrochloride for 30 min at 37°C[1]. The released scaffolding protein was then separated from the coat protein shells by passage through a gel filtration column (Fig. 4). Column fractions were assayed by electrophoresis through SDS polyacrylamide gels followed by Coomasie blue staining. The scaffolding protein eluted in a single broad peak. The fractions were pooled and applied to a DEAE cellulose column. The peak of the scaffolding protein eluted at 85 mM NaCl. The scaffolding protein sample used in the experiments described below is shown in Fig. 5. Although small amounts of coat protein and gene 20 protein were present as minor contaminants the scaffolding protein was over 95% pure judged by Coomassie blue stain. The purified scaffolding protein remained soluble both at room temperature and in the cold in Tris buffer.

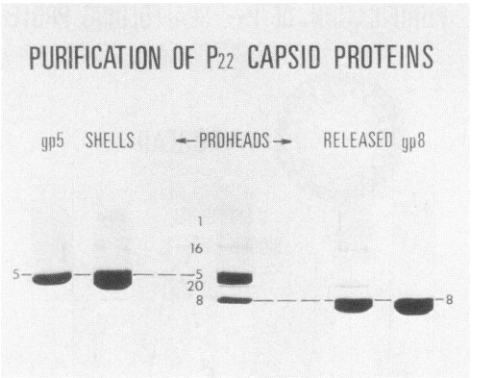

Figure 5 SDS gel analysis of purified coat and scaffolding proteins. Samples from various stages in the purification were dissociated into their constitutent protein chains by boiling in hot SDS and electrophoresed through a 10% SDS polyacrylamide gel. The central track shows the protein composition of the starting proheads. The next track to the right shows the scaffolding protein recovered from the Biogel column (Fig. 4). The right-most track shows the final scaffolding protein after a second fractionation on a DEAE Sephadex column (Fuller and King, manuscript submitted for publication). The empty coat protein shells and dissociated coat protein subunits, described in Fig. 4, have been electrophoresed through the left hand lanes.

Preparation of Coat Protein Subunits

The empty procapsid shells remaining after guanidine treatment consist primarily of coat protein. They also contain ~10–20 copies of the minor protein, gpl. This protein can be genetically removed by using procapsids isolated from cells infected with phage carrying an amber mutation in gene 1 as the starting material. The procapsids accumulating in such cells lack gpl (King et al., 1973).

The empty procapsids shells were dissociated into subunits by treatment with 3M GuHCl. When the resulting material was fractionated on a Biogel column run in 2 M GuHCl the dissociated coat protein subunits migrated as a single peak.[1] The protein was stored in 2 M GuHCl. For SDS gel analysis or biological experiments it was dialyzed against buffer B at 4°C to remove the guanidine. The coat protein remained soluble in the absence of guanidine in the cold. However, dialysis at room temperature or rapid passage through a desalting column to remove the guanidine resulted in formation of a flocculent precipitate. The coat protein preparation used for the experiments described below is shown in the left hand track of Fig. 5.

Electron Microscopy of the Isolated Subunits

Negatively stained preparations of the isolated coat protein subunits revealed no morphologically distinctive species. Aggregates of protein subunits were common but did not reveal any obvious organization.

The scaffolding protein was somewhat more difficult to visualize in the electron miscroscope. However, when we were able to identify fields containing stained protein, ring structures were the dominant species. A field of such structures are shown in Fig. 6. The dimensions are consistent with multimers of the scaffolding protein subunit. These aggregates may be induced by the high salt or low pH of the staining conditions, since we have been unable to find such multimeric species by centrifugation (see below).

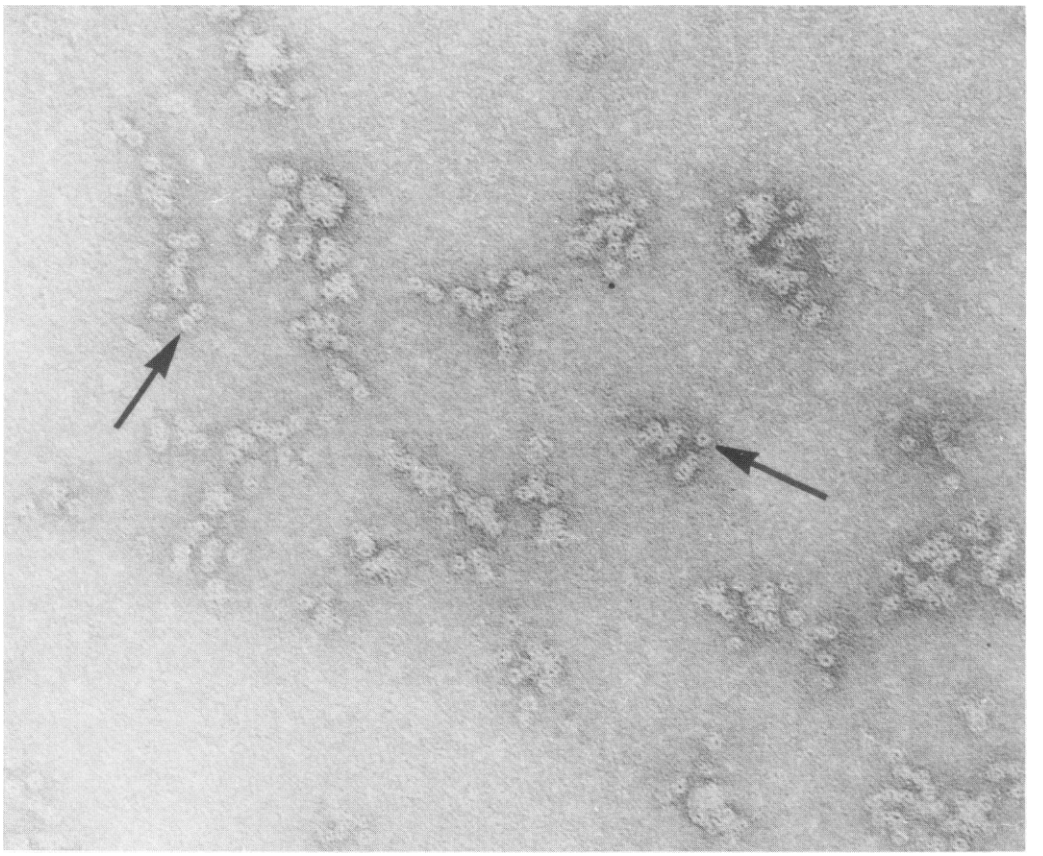

Figure 6 Electron micrographs of a scaffolding protein preparation. Samples of scaffolding protein were negatively stained with uranyl acetate. Note the ringlike structures. The protein in this sample was from the peak fraction of the scaffolding protein recovered from the gel filtration column. The protein had been dialyzed to buffer B to remove residual GuHCl. As noted in the text we were unable to identify scaffolding protein multimers by sucrose gradient centrifugation. We suspect that these complexes may have been induced by the low pH (4.5), high salt conditions of the uranyl acetate stain. However, within the organized procapsid, scaffolding protein subunits are presumedly complexed with each other. Thus, even though the protein is present primarily as monomer in buffer B, cyclic multimers such as those shown above probably represent physiologically important states.

In Vitro Reassembly of Prohead-like Particles from Purified Coat and Scaffolding Protein

To enable us to study the shell assembly reaction directly, we attempted to find conditions under which the purified proteins would reassemble into procapsid shells. Reassembly of procapsids was achieved by mixing together the coat and scaffolding subunits in the presence of GuHCl (final concentration of 1.5 M) and dialyzing the guanidine hydrochloride away overnight. Fig. 7 *a* shows an electron micrograph of the reaction mixture after dialysis against buffer B at 23°C. Particles closely resembling procapsids are common. Also visible in the field are a few particles which appear to contain internal material, but which are not of the correct dimensions.

Fig. 7 *b* shows an electron micrograph of the same protein mixture, for which dialysis took place at 4°C, rather than 23°C. Under these conditions, very few procapsid like particles were

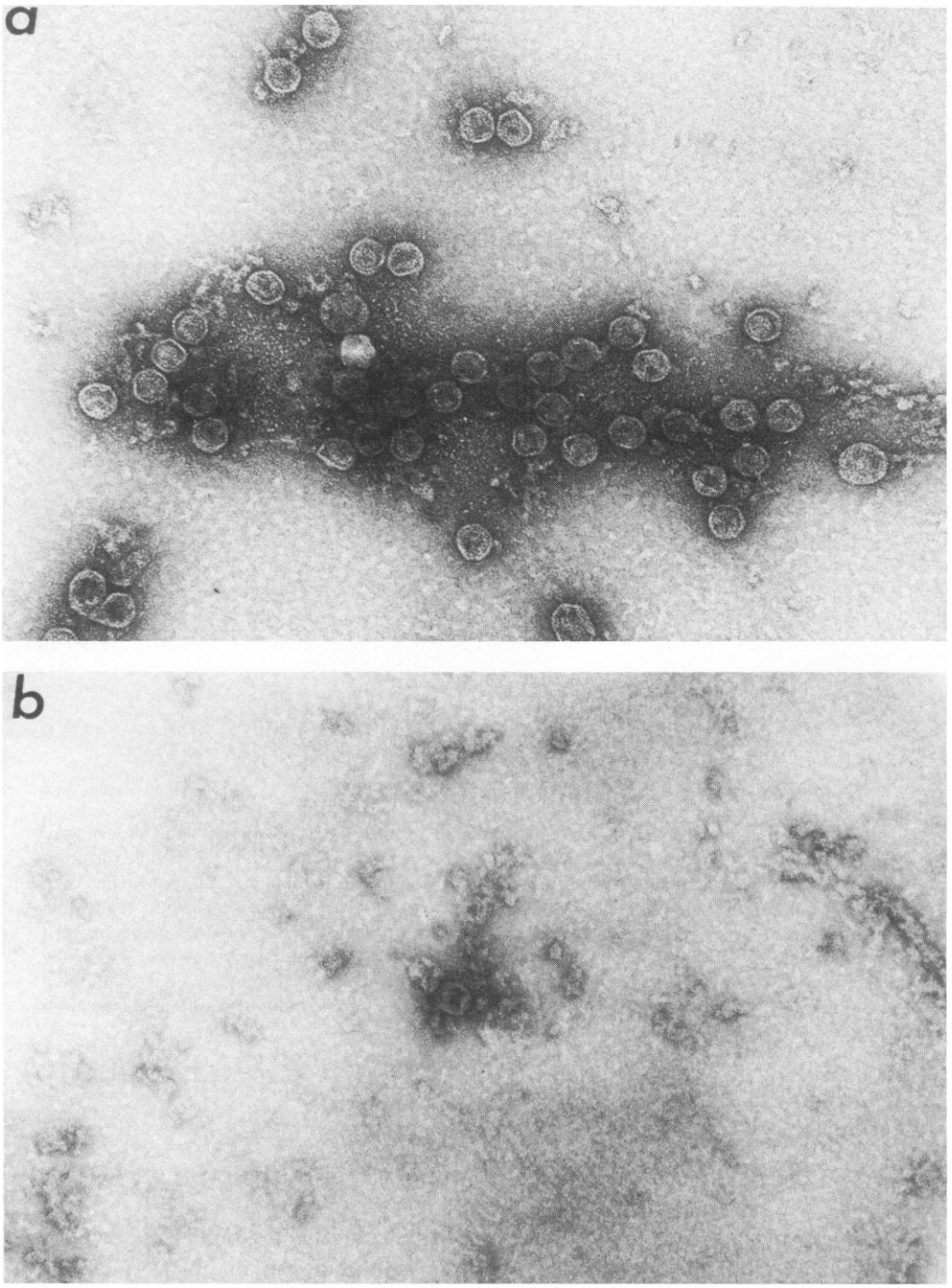

Figure 7 Electron microscopy of prohead-like structures formed in vitro. A 300 μl sample of coat protein in 2 M GuHCl in buffer B was mixed with 100 μl of scaffolding protein in buffer B. The mixture (1.5 M GuHCl) was dialyzed overnight either at 23°C (*a*) or 4°C (*b*). Drops of the reaction mixtures were then applied to carbon coated electron microscope grids and stained with 2% uranyl acetate. Most of the particles in *a* are indistinguishable from normal procapsids. A number of misshapen or aberrant shell structures are also evident. Very few particles of any type were found in the protein mixture that had been incubated at 4°C. The bar represents 0.1 microns.

observed. The fields contained protein aggregates, resembling those seen in negatively stained fields of pure coat protein. Though not visible in this micrograph, ring like aggregates similar to those shown in Fig. 6 were also occasionally seen.

To characterize the products of the in vitro incubation quantitatively, the samples were centrifuged through sucrose gradients to separate shell structures from unassembled subunits. The resulting fractions were analyzed by SDS gel electrophoresis to determine their protein composition. The results of this analysis are shown in Fig. 8. When the scaffolding protein (gp8) was dialyzed to buffer alone, all the protein remained at the top of the sucrose gradient as slowly sedimenting subunits. The coat protein (gp5) also remained as slowly sedimenting subunits when incubated alone, as shown in Fig. 8. However, when the two proteins were

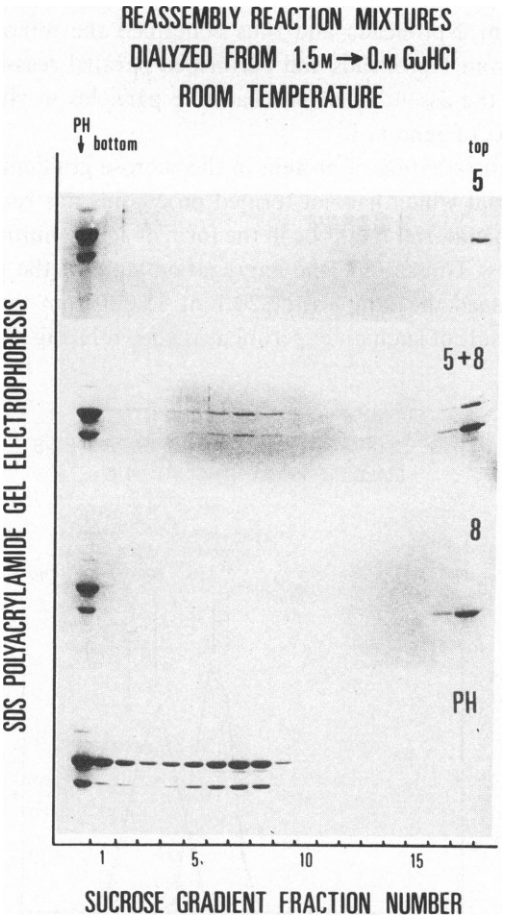

Figure 8 Sucrose gradient analysis of in vitro assembly reactions. Coat protein alone, coat protein and scaffolding protein, and scaffolding protein alone were made up to 1.5 M in guanidine hydrochloride. The GuHCl was removed by dialysis overnight at 23°C. 100 μl of each sample was then layered on a 5 ml 5–20% sucrose gradient (with a 0.3 ml 60% sucrose cushion) and centrifuged in a Beckman SW50.1 rotor. Gradients were centrifuged for 40 min at 35,000 rpm at 20°C, then fractions were collected and analyzed by SDS gel electrophoresis. Direction of sedimentation was from right to left. The left-most lane in each gel contains a sample of proheads to help identify the mobilities of the coat and scaffolding proteins in the gel. (5) Coat protein alone. (5 + 8) Coat plus scaffolding protein. (8) scaffolding protein alone. (PH) Control proheads isolated from 2^- infected cells.

incubated together and dialyzed, structures formed which sedimented in the center of the gradient. These structures have a ratio of coat to scaffolding protein which is characteristic of P22 procapsids. The bottom panel shows the sedimentation pattern of a more concentrated preparation of control procapsids formed in vivo. The peak of the distribution is in fractions 7 and 8. The procapsids formed in vitro sedimented at the same position in a parallel gradient.

The assembly reaction did not proceed efficiently if the dialysis was performed at 4°C. Most of the protein, even in the mixture, remained unassembled. At much higher protein concentration, the coat protein did aggregate in the absence of the scaffolding protein. The structures formed were small shells and spiral structures which closely resembled the aberrant aggregates formed in vivo in cells infected with mutants defective in the scaffolding protein (Lenk, et al., 1975; Earnshaw and King, 1978).

The coat protein preparation used in the in vitro assembly experiments shown in Figs. 7 and 8 was purified from 2-proheads and thus contained the minor protein gpl. We have prepared coat protein from 1-proheads and performed parallel reassembly experiments with the same results. Thus the assembly of prohead-like particles in vitro in this system is not dependent on the product of gene 1.

Examination of the distribution of protein in the sucrose gradients shown in Fig. 8 shows quite clearly that material which has not formed procapsids has remained at the top of the gradients. However, this material might be in the form of small multimers such as pentameric or hexameric aggregates. To examine the aggregation state of the unassembled proteins in more detail, we centrifuged the samples for 20 h at 45,000 rpm to resolve small molecular weight material. The result of such an experiment is shown in Fig. 9. The sample shown was

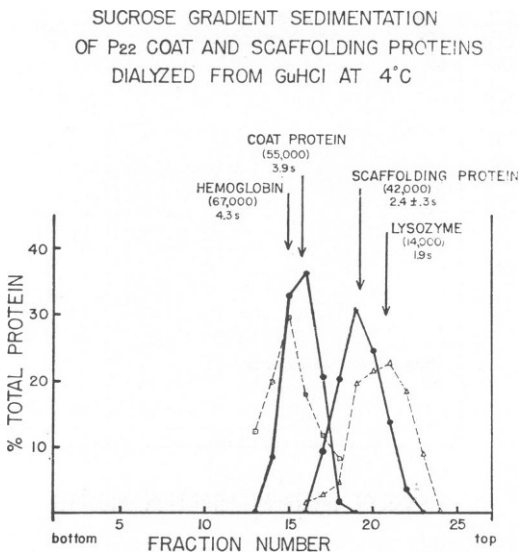

Figure 9 Sucrose gradient sedimentation of unassembled subunits. The mixture of coat and scaffolding protein described in the legend to Fig. 8 was dialyzed overnight against buffer B at 4°C. 100 μl of this material was mixed with 10 μl of hemoglobin solution (10 mg/ml) and 10 μl of lysozyme solution (10 mg/ml). The sample was layered on a prechilled 5–20% sucrose gradient and centrifuged for 20 h at 45,000 rpm at 4°C in a Beckman SW50.1 rotor. Fractions of 0.2 ml were collected and analyzed for their protein content by SDS gel electrophoresis. The gels were stained with Commassie brilliant blue and quantitated by tracing with a Joyce-Loebl microdensitometer.

the mixture of coat and scaffolding protein which had been dialyzed against buffer B at 4°C. Coat and scaffolding protein remaining unpolymerized after dialysis at 23°C exhibited similar sedimentation behavior.

The proteins sedimented independently of each other, with no evidence of interaction. Their sedimentation behavior, slower than hemoglobin, indicates that both species were sedimenting as monomers. The low sedimentation coefficient of the scaffolding protein, close to that of lysozyme, indicates that it is probably quite asymmetric, and is likely to be a long, thin or flat molecule.

DISCUSSION

In Vitro Assembly

The results described above indicate that the scaffolding and coat subunits activate each other in the shell assembly process. The scaffolding protein subunits alone showed no tendency to aggregate into large structures. Under the conditions of the experiment shown in Figs. 8 and 9, the coat protein also remained as soluble subunits. However, when incubated together, the two protein species assembled into structures with the same morphology and sedimentation coefficient as procapsids assembled in vitro. Since these structures also contained the coat and scaffolding protein in the same ratio as control procapsids, we presume they have the double shell structure of the starting particles.

Using coat protein preparations five- to tenfold more concentrated than those reported here, we have observed coat protein aggregation in the absence of scaffolding protein. The structures formed resembled the small shells and aberrant aggregates formed by coat protein in vivo in the absence of scaffolding protein (King et al., 1973; Earnshaw and King, 1978). However, even in this case, the presence of scaffolding protein during dialysis from GuHCl resulted in the preferential assembly of procapsid-like particles containing both proteins rather than aberrant coat protein aggregates (unpublished experiments).

We have also performed reassembly experiments in which the proteins were first removed from guanidine and then incubated together in the absence of guanidine. Procapsid-like structures were formed but with lower efficiency than in the dialysis procedure. Thus guanidine hydrochloride appears to play some role in the assembly reaction. King et. al. (1978) proposed that in the infected cell the scaffolding subunits might interact with nascent coat protein chains coming off the ribosome. If this were the case, the guanidine might partially unfold the coat subunits, generating the reactive form for binding to scaffolding subunits. The fully folded subunits would be less active. Thus, those subunits which had not yet polymerized when the concentration of guanidine hyrdrochloride dropped below a certain level during dialysis would tend to remain unpolymerized. This might account for the slowly sedimenting free subunits remaining in the reaction mixture (Fig. 9).

We have not detected large aggregates of scaffolding protein alone. Van Driel and Couture (1978) reported that the assembly core protein of T4 could form core-shaped structures in the absence of the coat protein. They proposed that these strucutres formed first and served as substrate for the polymerization of the coat protein. We cannot rule out the possibility that such scaffolding protein cores may have formed in our in vitro assembly reaction and were either converted to capsids in the presence of coat protein, or were unstable and dissociated into free subunits. As described below, we prefer a copolymerization model or vernier model (Paulson et al., 1976).

Subunit Arrangement

Casjens (1979) has analyzed the surface lattice of P22 phages and procapsids by electron microscopy of freeze-fractured samples. He proposed that the coat subunits have two domains, one of which interacts to form five- and sixfold morphological units, and the other of which interacts to form threefold units. The surface lattice of procapsids and phage were similar, indicating that the scaffolding subunits did not protrude. Casjens proposed that the coat protein had a hinge between the two domains (see also Harrison, this volume) which opened to generate the shell expansion associated with the transition of procapsid to mature capsid.

The scaffolding subunits are presumably themselves icosahedrally packed and symmetrically related to the coat subunits. If they are to exit through openings in the coat protein lattice they are likely to be located at gaps between subunits. There are ~200 scaffolding protein molecules and 420 coat protein subunits in the procapsid (King and Casjens, 1974). A packing arrangement that satisfies both the stoichiometry and symmetry considerations locates the scaffolding subunits at the twofold axes of the coat protein lattice (Fuller, 1979). This is shown in Fig. 10 for the outer surface of the shell. The scaffolding molecules extend inward to the center, where they pack with pentamer-hexamer clustering.

Pathway of Subunit Polymerization

In general, the assembly of bacteriophage procapsids in vivo initiates from a unique fivefold vertex, which also serves as the site for DNA packaging, and later for tail attachment and DNA ejection (Murialdo and Becker, 1978). It therefore seems reasonable to assume that the assembly of procapsids in vitro also initiates through formation of a fivefold vertex. Since procapsid assembly depends on the presence of both species of proteins, we envision that the initiation structure is composed of a pentamer of scaffolding protein bound to a pentamer of coat protein.

Assembly of prohead-like double shelled particles proceeds in the absence of gpI both in vivo (Botstein et al., 1973; King et al., 1973) and in vitro. Therefore gpI cannot be absolutely required for the initiation of prohead assembly. However, if gpI is normally located at the proximal vertex of P22 procapsids, like gpB of λ and gp20 of T4 (Murialdo and Becker, 1978) it may be incorporated into the initiation complex to ensure its proper localization in the completed particle. Location of gpI at the proximal vertex would also designate a unique vertex for DNA packaging. Therefore, in the model shown in Fig. 10, we propose that a complex of gpI with fivefold rotational symmetry is incorporated in place of the coat protein into the initial fivefold vertex in a wild type infection.

The observation that the scaffolding subunits alone do not self-assemble suggests that coat and scaffolding subunits add alternately to the growing shell structure. Before examining this in more detail, we need to enumerate the different probable states of the two subunits. The coat subunits can exist (*a*) free in solution, gp5°; (*b*) bonded to each other and to scaffolding molecules in procapsids, gp5′; and (*c*) strongly bonded to each other only, gp5″. Two states appear adequate to describe the life history of the scaffolding protein, (*a*) soluble subunits, gp8°, and (*b*) bonded to each other and to coat subunits in the prohead, gp8′.

precursors		procapsids		mature phage
gp5°	→	gp5′	→	gp5″
gp8°	⇌	gp8′		

Note that in the normal life history of the virus the coat subunits pass through a series of essentially irreversible conversions (Showe and Kellenberger, 1976). There is no reason to

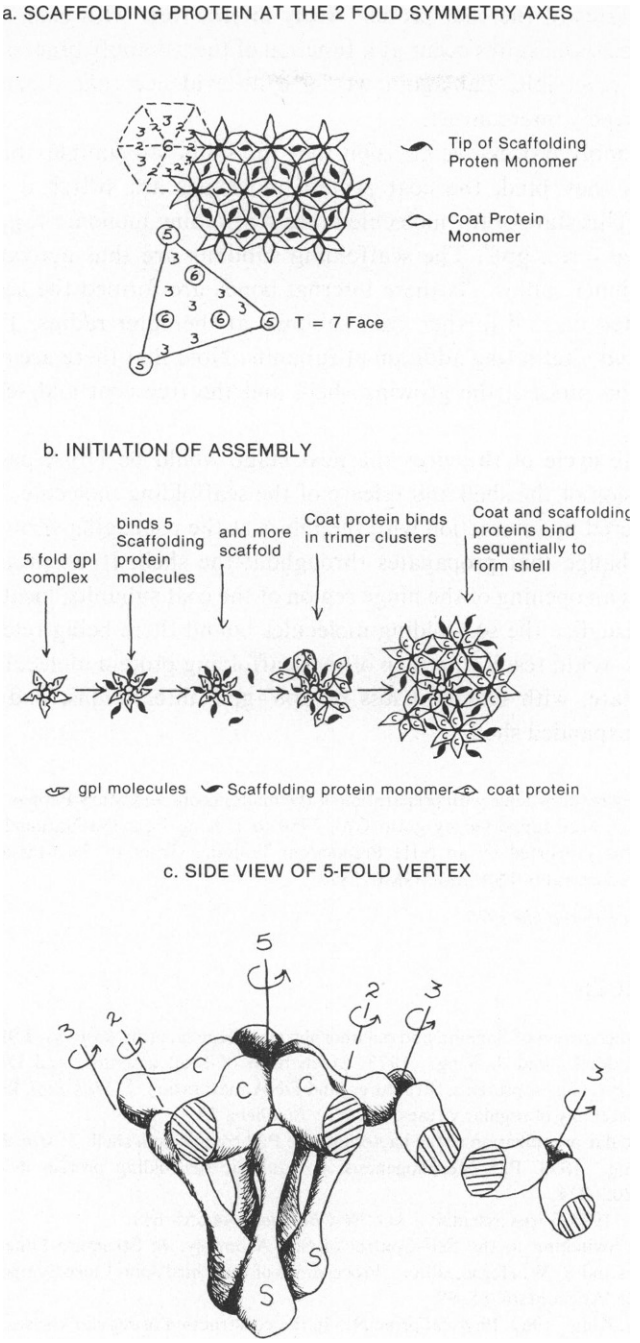

Figure 10 Topology and pathway of coat and scaffolding protein interaction. The drawing in (*a*) shows the packing of gp5 and gp8 most consistent with their stoichiometry in the procapsid, approximately one molecule of gp8 for two molecules of gp5 (King and Casjens, 1974). The arrangement of coat protein on the surface of P22 proheads is based on the work of Casjens (1979). (*b*) shows a possible in vivo pathway for initiation of shell assembly in which gpl forms the first fivefold vertex. Our in vitro results show that gpl is not absolutely required for shell assembly; we presume that in vitro five coat and five scaffolding protein subunits form the initiation complex. However, we know that several molecules of gpl must be incorporated into the procapsid during its assembly to form infectious virus (Botstein et al., 1973; King et al., 1973; Poteete et al., 1979). By analogy with T4 and lambda, these molecules are probably located at the initiating vertex (Murialdo and Becker, 1978). One way to achieve this structural arrangement is to initiate assembly with gpl, as is the case with gp20 of T4. In T4, gp20 is required for initiation of capsid assembly. In P22, gpl is not required for initiation. (*c*) shows a side view of a possible arrangement of the coat and scaffolding proteins at the inner and outer radii of a fivefold vertex.

believe that these states of the coat protein freely interconvert with each other in solution. Instead transitions between states occur as a function of the assembly process. The scaffolding transition must be reversible, but again we have no evidence that the two states of the scaffolding protein freely interconvert.

In the shell assembly process we envision that the initiation complex binds the first few gp5° molecules. As they bind, the coat protein molecules are switched into the reactive conformation gp5′. This state of the molecule binds scaffolding monomers, gp8°, and switches them to the reactive form, gp8′. The scaffolding subunits are thus activated for gp8′-gp8′ interactions at the inner radius. As these internal bonds are formed the scaffolding protein subunits are activated to bind further gp5° subunits at the outer radius. This would assure strictly sequential and alternating addition of subunits. Note that there are only three species in the polymerization process: the growing shell, and the free coat and scaffolding protein subunits.

In the normal life cycle of the virus the next stage would be DNA packaging, with its concomitant expansion of the shell and release of the scaffolding molecule. We envision that this process is triggered by interaction with the DNA at the packaging vertex, which includes a conformational change that propagates throughout the shell. If, as proposed by Casjens (1979), this involves an opening of the hinge region of the coat subunits, located at the twofold axes, it is easy to visualize the scaffolding molecules bound there being released. The loss of the gp5′–gp8′ bonds would result in return of the scaffolding protein molecules in the prohead core to the gp8° state, with resultant loss of gp8′–gp8′ interactions, and diffusion of the subunits out of the expanded shell.

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DISCUSSION

Session Chairman: Alan Schechter *Scribe*: Pieter De Haseth

BUTLER: On the electron microscope grids you do see aggregates of the scaffolding protein. This reminds me of an observation that Ken Adolph and I made some years back with Cowpea Chloric Mottle virus protein. Under conditions where the protein would not assemble at all in solution, we were finding a hexagonal net of the protein on the surface of the electron microscope grids. At the time, I ascribed this to the fact that there was a nucleation barrier which could be overcome if the protein were interacting with the RNA. This is only a hypothesis, but it could be that the carbon substrate of the EM grid might be overcoming the barrier. It would be very interesting if the interaction of the coat protein with the scaffolding protein overcame the same sort of nucleation barrier, and in fact the parallel is closer than I realized at the time.

FULLER: That is precisely the kind of thing we would like to invoke to explain our results. In electron micrographs we see aggregates of the scaffolding protein, some of which clearly look like pentamers. The same sample sediments as a monomer in sucrose or D_2O gradients. We think the protein exists as a monomer in solution, and, as you pointed out, that the conditions used for EM visualization induce aggregation. In addition to the possible role of interaction, aggregation may have been induced by the high salt or low pH of the uranyl acetate stain. Under these conditions,

aggregates may form which may normally arise only during the interaction between the coat and the scaffolding protein. The fivefold complexes of scaffolding protein visualized in the electron microscope may thus indeed be related to the biological reaction.

VAN HOLDE: I have a suggestion that might get away from having to deal with an activation state for the scaffolding protein and also might possibly explain the effects on the microscope grid.

If the scaffolding protein were to have a very weak self-association reaction with a small equilibrium constant, then once they became stuck to the coat protein their local concentration would be increased by orders of magnitude compared to that in dilute solution. Then they could self-associate. It could be that in solution at that high concentration they would also show this behavior. So one does not need to say that they have to be activated.

KING: I agree. The only problem with that is if you invoke local concentration of the scaffolding protein as a reason for not seeing any self-association, it is hard to understand why the scaffolding protein would ever come out of the prohead, because in any form of the shell these molecules are very close together.

If the reason they self-associate inside the shell is because of a small equilibrium constant, then it is hard to explain why any change of the conformation of the outer shell would let those 200 mol go out. You have to give the system some property, so that there is a state in which the scaffolding molecules do not bind to each other.

VAN HOLDE: But, as I understand it, you do special things in order to get them out. Either you change the solvent conditions, or you put some DNA in.

KING: You are right that *in vitro* we have detergent or GuHCl present, so we perturb the system. But *in vivo* we have no reason to think that in a five min span of time the ionic conditions inside a Salmonella cell are changing. It is true that the DNA is being inserted into the particle but presumably the scaffolding protein has to leave first, because it occupies the internal volume. We prefer a model where you trigger a change in the coat protein in order to drive the release of scaffolding protein.

FULLER: We must begin to grapple with what might happen if instead of one protein assembling, as is the case for TMV, we have two proteins interacting and controlling each other's assembly. As yet there is no coherent thermodynamic theory which adequately describes this kind of two-protein assembly system.

KAHN: I would like to propose the "battleship" explanation for how the scaffolding protein gets out. Professor Crane, a well-known physicist who became a biologist towards the end of his career, used to recount that during World War II a battleship hull was being put together at the Brooklyn Navy Yard drydocks. When they put in the last few plates the entire hull cracked open from bow to stern. Crane took models of this kind to show how the completion of a large structure could lead to a sudden increase in stress and thereby to a massive reorganization. The sudden exit of the scaffolding protein could arise from a completion of the prohead, leading to its suddenly opening up.

FULLER: The problem with that model is that P22 proheads are very stable. They can sit at 4°C for a year after being purified and nothing happens.

KAHN: But that's after they come out of the cell.

FULLER: Proheads are also stable in the cell. They accumulate in cells infected by phage carrying a mutation which inactivates one of the minor proteins needed for DNA packaging. Therefore one cannot evoke completion of the shell as the signal for the exit of the scaffolding protein.

VON HIPPEL: About the chronology of events, do you find that the scaffolding protein only starts leaving when the DNA starts arriving? Secondly, this whole expansion must leave enormous holes. How big a thing can you get into the structure and touch the DNA with after you have finished extruding the scaffolding protein?

FULLER: Thus far, for P22 we have not been able either genetically or biophysically to dissect the events which accompany DNA packaging. There are three events: (*a*) The scaffolding protein exits from the prohead; (*b*) The coat protein shell expands by ~10% of the radius; (*c*) The DNA is packaged.

KING: As far as the "holes" in the head are concerned, it looks like it is the other way around. The precursor shell has holes big enough for a protein of MW 40,000 to exit. For the shell rearrangement, we would invoke a Harrison-type model in which each coat protein has a hinge and each subunit would change the angle of its hinge. After the ensuing expansion the capsid is much more tightly packed than the precursor capsid. For the precursor, addition of a little detergent will make the subunits fall apart; for the phage, you have to heat to 100°C, with

detergent, to get the subunits apart. We imagine movement which fills up the holes and alters the shell from a DNA packaging to a device which protects the DNA from nucleases.

VON HIPPEL: Can you get something into the phage?

KING: You can get in acridines, but only slowly, so the holes are not vast. Aflatoxin, which is a bigger ring-structure, will not go in.

MAKOWSKI: How do you know the scaffolding protein is on the inside?

FULLER: The clearest evidence was obtained by low angle x-ray diffraction by Dr. Earnshaw in collaboration with Dr. Harrison. Their findings are summarized in our Fig. 3. Compare the distribution of density in proheads, which contain the scaffolding protein, with empty heads, which have lost their scaffolding protein and have expanded to the mature radius. Proheads have a thin outer shell which surrounds an internal core of dense material. The empty heads, which contain no scaffolding protein, have a thin outer shell but no internal density. From this we argue that the scaffolding protein was on the inside.

MARTIN: Is it possible that the whole thing folds inside out?

KING: That would be the "bathing cap" model. It is technically possible. However, a graduate student of mine, Ruth Griffin Shea, was able to catch intermediate stages of the process with antibody against the scaffolding protein. Antibodies against the scaffolding protein coated the capsid, and instead of having the inner part of the shell filled with protein, it was stain-penetrated, as if the antibodies had caught the scaffolding subunits going out and bound to them. In such pictures the coat protein shell remained intact. It did not appear to be turning inside out.

In T4 one gets rid of this problem in a much easier way. During T4 capsid assembly, a protease is incorporated into the head. This protease is activated after completion of prohead assembly. It then hydrolyses the scaffolding protein into small peptides which can diffuse out much more easily, although some actually stay in. Each virus has a different mechanism for solving the problem.

There could be a limit to the accuracy with which the volume of a shell, consisting of hundreds of subunits, can be specified. The volume is very important, because it determines the size of the genome, as these viruses package by headfulls.

STEVEN: I want to caution against drawing global conclusions from negative results of assembly reactions. One tries a limited number of conditions *in vitro*, and then extrapolates the negative results obtained to the conditions inside the cell. Recall Van Driel's published results on the successful assembly *in vitro* of T4 prohead cores in the absence of capsid protein. The structures produced are morphologically convincing in comparison to electron micrographs of core-containing proheads. These results may also imply that analogies between the assembly properties of the different double-stranded DNA phages extend only so far.

FULLER: It is not clear to me which of the two cases, T4 or P22, is going to be the universal one, if a universal mechanism exists. Van Driel's work was done at a much higher protein concentration —as much as 50-fold higher—than our work. At high protein concentrations, the P22 coat protein assembles into aberrant structures. The scaffolding protein may do so as well.

I am unaware of any evidence from Van Driel's work that core structure assembled from the T4 scaffolding proteins is actually an intermediate in the assembly and not a side pathway. Are you familiar with any such evidence?

STEVEN: I don't know of any direct evidence that Van Driel has produced, but I have seen unpublished results quoted in the annual report from that laboratory, by Kellenberger, Traub, and coworkers. In bacteria infected with mutant T4, defective in the production of the major capsid protein, they report particles lined up along the inner surface of the cytoplasmic membrane that morphologically have the correct dimensions and shape, within the very limited resolution available with that sort of thin section.

FULLER: That is very interesting. Further experiments are required to establish a precursor-product relationship. A number of mutants in the capsid protein of T4 have been known to make phages with heads which are too long or too short. Thus it seems to me that the capsid protein also must take part in the determination of capsid size.

Revertants to such capsid-size mutants which map the T4 core protein have been isolated. This genetic evidence implicates an interaction of the coat and scaffolding in size determination.

Thus in T4 it is not yet clear whether the core is assembled first, and the coat protein laid around it like shingles on a roof, or whether the coat protein and core particles copolymerize.

STEVEN: We could discuss that later, but I want to make one last comment. It was mentioned earlier that multiple pathways for assembly may exist. I would expect them to be more likely in a relatively complicated system like T4 or P22, where there are many proteins involved, rather than in a simple RNA virus, where there are relatively few different proteins.

FULLER: Indeed, the coat proteins of P22 and T4 can make a large number of different structures, most of which are dead ends. Probably there is only one pathway that leads to an infectious virus. Specific control mechanisms must exist which assure that the correct assembly pathway is taken. The interaction of the coat and scaffolding protein of P22 is one such control mechanism.

KING: Most notably in Caspar's presentation one could see clearly that there were structures with fivefold axes and sixfold axes of symmetry made with the same subunits. It is not always pointed out that no mechanism is yet known to instruct the subunit that comes in to go fivefold rather than sixfold. This information is not built into the coat protein subunit of P22. The coat protein by itself gives rise to a variety of aggregates which don't have the fivefolds and sixfolds correctly located. The interaction between the coat protein and the scaffolding protein must somehow instruct the subunit that is coming in to five-coordinate or six-coordinate. This is not a thermodynamic or statistical phenomenon; it is absolutely precise in the cell.

WETLAUFER: I should like to raise an objection to your assigning an enzyme activity to the scaffolding protein. I understand why you made that assignment, but I am afraid that it is a rather peculiar enzyme that has to have this particular stoichiometric relationship with the coat protein before it is recycled.

FULLER: We would call it a stoichiometric catalyst.

KING: Such assembly catalysts may be quite common in assembly processes, but one does not find them if one does not look for them. Since the assembly proteins are not present in the final structure one sees them only if one can accumulate the intermediates. There is now evidence in the adenoviruses for the same kind of scaffolding protein. We should assign a name to this class of molecules.

BLOOMFIELD: In the electron micrographs of your reconstituted double shells (Fig. 7) I thought I saw an occasional little tail sticking out. This might be a special vertex protein, yet I would have assumed that that was a pure gene 5 and gene 8 combination. Do you have any comments on what that little projection is?

FULLER: The electron micrographs show the entire reaction mixture which contains a significant amount of unassembled protein. The occasional "projecting structure" is most likely unassembled protein sitting by a prohead.

BLOOMFIELD: Are these reassembled heads competent to accept DNA?

FULLER: No, they are not. Fig.1 shows the reason why. There are four minor proteins (gp1, gp16, gp20, and gp7) built into a normal prohead. In order to get infectious phage, these minor proteins have to be built into the prohead and cannot be added afterwards. The product of gene 1 is required for DNA packaging; gp16, gp20, and gp7 are required for DNA injection on the next round of infection. The particles assembled *in vitro* were assembled from purified coat and scaffolding protein. Because they lack the minor proteins, the proheads could not package DNA and mature into infectious phage.

In our paper we did not include our experiments using a DNA packaging system for P22 delevoped by Poteete and Botstein. This system consists of a crude extract made from cells infected with phage which have a mutation in the coat protein. The infected cells fill up with P22 DNA and all the other proteins required for virus assembly except for the coat protein. There are no heads assembled. Addition of proheads to the extract will, in the presence of ATP, result in the packaging of DNA into the proheads followed by maturation and tailing, resulting in the production of infectious virus.

When we add purified, unassembled coat protein and scaffolding proteins to the extract, instead of proheads, the extract provides the four minor proteins, and the coat and scaffolding proteins assemble into active proheads which package DNA and mature into infectious phage.Thus we do have an activity assay for the purified proteins.

KING: Actually, you did put your finger on what is a major fudge in our paper. The P22 shell, like every viral shell, has a unique vertex. All the evidence indicates that this is the vertex that initiates capsid assembly. Although the purified coat and scaffolding proteins can assemble a shell *in vitro* without a specialized vertex protein, we believe that inside the cell a special vertex containing the minor protein gp1 initiates. In T4 you cannot build a prohead unless you have a special vertex protein.

BLOOMFIELD: It has been suggested that scaffolding protein may have an entirely different function, which is to exclude other cellular protein from the interior of the capsid. Would you like to comment on that?

KING: That is a good point. Inside a Salmonella cell, where the protein concentration is very high and no compartmentalization operates, the capsid would capture a lot of proteins, including nucleases. Also, one knows from work with T4 that the DNA has all kinds of proteins on it: replicases, recombination enzymes, repair enzymes, and unwinding proteins. The DNA packaging process must involve special proteins that peel off those enzymes. If the phage ingest those kinds of proteins it seems feasible that the scaffolding proteins exclude the garbage from the inside of the virus.

FULLER: We should not lose sight, however, of the fact that there is sound genetic evidence that the scaffolding protein serves a function in determining the curvature of the shells. Both *in vitro* and *in vivo*, without scaffolding protein the coat protein makes shells that are too small and spiral structures which do not close, as well as shells of the correct size.

KING: Dr. Bloomfield, you know that if you are going to put up a good brick wall you've got to have scaffolding, right?