Assembly of T7 Capsids from Independently Expressed and Purified Head Protein and Scaffolding Protein

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Prohead-like capsid shells containing the scaffolding and head proteins of bacteriophage T7 were isolated after both proteins were expressed from the cloned genes in the same cell. When the head–tail connector protein was also expressed, the isolated capsids contained neither connector nor scaffolding protein and resembled mature phage capsids rather than proheads. However, only a small fraction of the head protein was converted to stable capsid structures in either case. Purified scaffolding protein (expressed individually from the cloned gene) appeared to be a monomer in solution; purified head protein appeared to be a tetramer. The purified proteins reacted in the presence of polyethylene glycol or dextran to produce prohead-like capsid shells and also polycapsids consisting primarily of head protein, similar to the polycapsids observed after infection by T7 mutants lacking connector or core proteins. Neither capsids nor polycapsids were produced in the absence of scaffolding protein. Polycapsids were usually the predominant product even when scaffolding protein was in excess, and a small fraction of scaffolding protein catalyzed the conversion of an excess of head protein to polycapsids. Our results suggest that the first step in the natural pathway to prohead formation is the assembly of incomplete prohead shells, which are normally closed by insertion of a connector–core complex. In the absence of a functional connector–core complex, incomplete capsid shells apparently react further to form polycapsids or completely closed capsid shells.

Keywords: bacteriophage T7; capsids; assembly from purified proteins

Introduction

Bacteriophage T7 provides a well defined system in which to study the early steps of head assembly and the possible roles of the different proteins involved. The phage particle contains a linear 40 kb double-stranded DNA packaged inside a symmetrical polyhedral protein shell about 60 nm across, a short tail, and an internal core structure that is coaxial with the tail (reviewed by Steven & Trus, 1986). The entire sequence of T7 DNA is known (Dunn & Studier, 1983), T7 proteins are readily expressed from the cloned genes (Studier & Moffatt, 1986), and the structure, protein constituents and assembly pathway of the phage particle have been studied in some detail (Studier, 1972; Serwer, 1976; Roeder & Sadowski, 1977; Serwer & Pichler, 1978; Masker & Serwer, 1982; Steven et al., 1983; Serwer et al., 1983).

As with other bacteriophages that contain double-stranded DNA, initial assembly of the protein shell requires not only the head protein but also a second protein, referred to as the scaffolding protein, which is found in proheads but not in DNA-containing particles (reviewed by Earnshaw & Casjens, 1980; Bazinet & King, 1985; Casjens & Hendrix, 1988; Black, 1989). The initial prohead shell undergoes a structural transformation and expansion upon loss of the scaffolding protein and packaging of the DNA. Both proheads and mature phage incorporate a structure into the capsid shell called the connector or portal, through which DNA
enters the head during packaging and leaves the head during entry into the host cell, and to which the core and tail structure attach.

In T7, the connector protein is specified by gene 8, the scaffolding protein by gene 9, and a pair of overlapping head proteins by gene 10 (Studier, 1972; Serwer, 1976). The major head protein gp10A contains 344 amino acids; the minor head protein gp10B contains 397 amino acids and arises by a translational frameshift at Phe341 of gp10A (Dunn & Studier, 1983; Condron et al., 1991). T7 mutants lacking the scaffolding or head proteins are unable to produce capsids (Roeder & Sadowski, 1977; Serwer et al., 1982), and those lacking connector, scaffolding or head proteins are unable to process concatemeric replicative DNA to form mature phage DNA (Hausmann & LaRue, 1969; Studier & Hausmann, 1969). Mutants lacking the connector protein or any of the proteins of the internal core (specified by genes 14, 15 and 16) are able to make capsids but also accumulate aberrant, typically tubular capsid-related structures referred to as polycapsids (Roeder & Sadowski, 1977; Serwer, 1979; Steven et al., 1983).

Prohead shells of bacteriophage P22 have been assembled from scaffolding and head proteins that were purified and renatured after dissociating them from proheads with guanidinium hydrochloride (Prevelige et al., 1988), and prohead shells of bacteriophage φ92 have been assembled by co-expression of connector, scaffolding and head proteins from the cloned genes in Escherichia coli (Guo et al., 1991). Connector protein was not needed for assembling uniform P22 particles from renatured scaffolding and head proteins, but in vivo assembly of scaffolding and head proteins of φ29 without the connector produced particles that varied in size and shape.

We have explored possible roles of the connector, scaffolding and head proteins in T7 capsid formation. We first looked for capsid-related structures after expressing different combinations of the cloned genes in the same cell and then analyzed reactions between proteins that had been purified after expression from the individual cloned genes. These proteins were not exposed to other components of normal phage particles or to T7 proteins that might have an accessory role in capsid assembly. The purified proteins were not exposed to denaturants and were presumably in conformations similar to those in the natural assembly process.

# Results

## Capsids from co-expressed scaffolding and head proteins, with or without connector protein

Two types of empty heads are typically isolated after wild-type T7 infection (Studier, 1972; Serwer, 1976; Roeder & Sadowski, 1977), and these were used as standards in searching for capsids produced from proteins expressed from cloned genes. Proheads contain the products of genes 8, 9, 10, 14, 15 and 16 (the connector, scaffolding, head and core proteins) and are the normal precursors to DNA-containing phage particles. What we refer to as converted heads lack scaffolding protein, have the morphology typical of mature phage heads, and presumably arose from abortive attempts to package DNA into proheads or from unstable particles that released their DNA. The two types of empty head resolve well upon electrophoresis through agarose gels, with proheads having a much higher mobility, presumably due to the highly acidic scaffolding protein (Serwer & Pichler, 1978; Dunn & Studier, 1983; Figure 1b).

No capsids nor any discrete structures made of head protein were isolated by standard purification procedures from extracts of cells in which the head protein was produced from the cloned gene in the absence of scaffolding protein. On the other hand, extracts of cells in which both scaffolding and head proteins had been expressed generated small amounts of what we refer to as 9-10 heads. The 9-10 heads migrated close to proheads upon agarose gel electrophoresis (Figure 1b), had the appearance of prohead shells in conventional electron micrographs (Figure 2), and appeared to contain scaffolding and head protein in approximately the same ratio as in proheads, as determined by electrophoresis through polyacrylamide gels in the presence of sodium dodecyl sulfate (Figure 1c, and others not shown). The 9-10 heads appeared to contain only a small fraction of the scaffolding and head proteins produced in these cells. Most of the scaffolding protein but little of the head protein remained in the soluble fraction of the extracts. A white precipitate that was mostly head protein, with at most a trace of scaffolding protein, was found in the CsCl gradient at a lower density than the 9-10 heads. This precipitate was not apparent when head protein was expressed in the absence of scaffolding protein. Although this material was not examined in the electron microscope, it seems likely to have consisted of polycapsids, in light of the subsequent in vitro results described below.

Expression of connector in addition to scaffolding and head proteins generated small amounts of what we refer to as 10-heads. The 10-heads migrated close to converted heads upon agarose gel electrophoresis (Figure 1b), had the appearance of converted heads in conventional electron micrographs (Figure 2), and appeared to contain only head protein and no connector or scaffolding protein when analyzed by gel electrophoresis in the presence of sodium dodecyl sulfate (Figure 1c). As with the 9-10 heads, the 10-heads, appeared to contain only a small fraction of the gp10 produced in these cells.

## Purified scaffolding and head proteins

Encouraged by the isolation of small amounts of capsid structures when scaffolding and head proteins were produced in the same cell, we expected that we could find conditions for assembly
T7 Capsids Assembled from Purified Proteins

Figure 1. Gel electrophoresis of purified proteins, intact heads, and dissociated heads. a, Head protein and scaffolding protein purified after expression individually from the cloned genes, and c, 10-heads isolated after co-expression of connector, scaffolding and head protein in the same cell, 9-10 heads isolated after co-expression of scaffolding and head proteins in the same cell, and proheads and converted heads isolated from wild-type T7 lysates were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. b, The same set of heads, without disruption, were analyzed by electrophoresis through a 1% agarose gel (pH 8.0).

Therefore, we expressed and purified these proteins individually from the cloned genes, as described in Materials and Methods. The purified scaffolding protein eluted at a position corresponding to a molecular mass of 49 kDa upon gel filtration chromatography in buffered 50 mM NH₄Cl, whereas the size calculated

Figure 2. Electron micrographs of proheads, converted heads, 9-10 heads, and 10-heads. Samples of a, proheads, b, 9-10 heads, c, converted heads, and d, 10-heads, described in more detail in the legend to Figure 1, were stained with 2% uranyl acetate and examined by conventional transmission electron microscopy (the bar represents 100 nm). The small round particles apparent in the 10-heads, and to a lesser extent in the converted heads, are believed to represent a cellular component that bands at approximately the same position as converted heads in the CsCl step gradients used. These particles do not have the characteristic appearance of T7 connectors (see Cerritelli & Studier, 1996) and the 10-heads contained little if any gp8 (Figure 1c).
Testing conditions for the assembly of T7 prohead shells from purified scaffolding and head proteins. Scaffolding protein (gp9) and head protein (gp10) were combined in 1:1 molar ratio, and aliquots containing approximately 6 μg of each were mixed with the indicated reagents at the indicated pH in a total reaction mixture of 12 μl. The buffer used in the pH range of 7.0 to 9.7 was 50 mM Tris-HCl, and at pH 6.0 was 50 mM Na2-Pipes. All reactions contained 40 mM NaCl and were incubated at 37°C for 15 minutes. A control reaction where water replaced the added reagents was included (a), along with a gp9 and a gp10 standard (a and c). Dextran T-10, sucrose, and glycerol were used at 20% final concentration, and ethylene glycol (EG) at 10% and 20%. PEG200 was used at the concentrations noted in a and c; the higher molecular weight PEG samples in b were at concentrations of 15%, 10% and 5% (from left to right). The reaction mixtures were electrophoresed on a 2% agarose gel (pH 8.0), and stained for proteins. The position of 9-10 heads isolated after co-expression of scaffolding and head protein in the same cell, is indicated by the arrow.

from the nucleotide sequence is 33.8 kDa (Dunn & Studier, 1983). It was noted (Studier & Maizel, 1969) that this protein migrates anomalously during electrophoresis through polyacrylamide gels in the presence of sodium dodecyl sulfate, changing position relative to other proteins as a function of gel concentration. Under conditions used in this work, it usually migrated to a position corresponding to greater than 43 kDa. The scaffolding protein seems likely to be a rather extended monomer in solution.

The scaffolding protein is quite sensitive to digestion by proteases during purification or to added trypsin or subtilisin, which produce a series of discrete degradation products (shown by Cerritelli, 1991). For this reason, a protease inhibitor was added to buffers during purification. Scaffolding protein is also very heat stable, remaining soluble after 12 minutes in a boiling water bath. It is one of the few proteins in an E. coli extract to remain soluble after this treatment, which could perhaps be used for rapid purification. It is highly soluble and has a high negative surface charge, as predicted from its amino acid composition (Dunn & Studier, 1983) and demonstrated by its tight binding to DEAE-Sepharose and its high mobility in non-denaturing agarose gel electrophoresis (Figure 3).

Initial attempts to purify head protein were unsuccessful, because extracts made under conditions where scaffolding protein was completely soluble produced head protein that was completely insoluble. Exploration of conditions for making extracts revealed that reducing the concentration of the extracts and including 0.1 M NaCl in the extraction buffer could produce extracts where essentially all of the head protein was soluble and could be readily purified. The two forms of head protein, gp10A and gp10B, co-purified through our procedures, which is not surprising because the two forms have their first 341 amino acids in common. The purified gp10AB mixture eluted at a position corresponding to a molecular mass of about 150 kDa upon gel filtration in buffered 0.5 M NaCl. This observation, combined with the predicted molecular mass of 36.4 kDa for gp10A and 41.7 kDa for gp10B (Dunn & Studier, 1983), suggests that the head protein forms tetramers under these conditions.

Conditions for assembly of prohead shells

Reaction mixtures containing purified head and scaffolding protein were analyzed for the formation of prohead shells, by electrophoresis on non-denaturing agarose gels under conditions where the head protein barely enters the gel, the scaffolding protein runs far into the gel, and any prohead shells would migrate to a position between them (Figure 3). To survey for conditions where prohead shells might form, mixtures of scaffolding and head protein were incubated for up to three hours under a variety of conditions, including temperatures.
between 0°C and 65°C, different ratios of the proteins, pH values between 5.0 and 9.7, different salt concentrations, and with additives such as 1 mM spermidine, 0.1 M or 1 M urea, or 2 mM ATP. At head protein concentrations around 1 mg/ml, none of these reactions produced any apparent capsid structures and the starting proteins appeared to remain completely unreacted. However, the head protein moved slightly farther into the gel in the presence of scaffolding protein, suggesting at least a weak association between them (e.g. the first two lanes of Figure 3 and the controls in Figure 6). At higher concentration, approximately 10 mg/ml of scaffolding protein and 5.5 mg/ml of head protein, most of the protein again remained unreacted, but a faint band was apparent at the position of 9-10 heads.

Reasoning that assembly of 9-10 heads during co-expression in *E. coli* might have been catalyzed by some factor supplied by the host cell, we made an extract from a mixture of cells in which the scaffolding and head proteins had been expressed separately. Indeed, 9-10 heads were isolated from such a mixture, but further investigation showed that the polyethylene glycol used to precipitate the heads as the first step in isolation was responsible for stimulating their formation. Addition of polyethylene glycol (PEG) or dextran T-10 to a reaction mixture containing only purified scaffolding and head proteins in buffered saline stimulated the production of a discrete band close to the position where 9-10 heads would migrate (Figure 3). Material that remained in or near the sample well may also have been produced but was difficult to distinguish from unreacted head protein under these conditions of electrophoresis.

The ability to stimulate capsid formation, as measured by the electrophoretic assay, was tested with different concentrations of PEG of molecular weight averaging 200, 600, 1450, 3350 and 8000 (Figure 3). The PEG concentration optimal for stimulating capsid formation increased as the polymer length decreased, from about 5% (w/v) for PEG8000 to about 10% for PEG3350, PEG1450 and PEG600, to about 20% for PEG200. Ethylene glycol, glycerol or sucrose at concentrations of 20% (w/v) did not stimulate any apparent reaction of scaffolding or head proteins. We used 20% PEG200 in further reactions because it seemed to stimulate the reaction as well as the larger polymers did, and its lower viscosity made it easier to work with.

Capsid formation in 20% PEG200 had a pH optimum between 7.0 and 8.2 (Figure 3c). The yield of capsids was highest at 40 mM NaCl, slightly lower at 80 mM NaCl, and strongly suppressed at 160 mM or 320 mM NaCl (Figure 4b). Incubation at 21°C and 37°C gave similar reaction products, but the yield of capsids was reduced at 0°C and 12°C, with a greater fraction of the product remaining in the sample well, particularly with PEG8000 (not shown). We settled on standard conditions of incubating at 37°C in 50 mM NaCl, 50 mM Tris-HCl (pH 8.2), 20% PEG200.

### Effect of the ratio of scaffolding to head protein

Proheads isolated after T7 infection contain about one-third as many molecules of scaffolding protein as head protein (Serwer, 1976), so reaction of a 1:1 molar ratio might be expected to leave as much as two-thirds of the scaffolding protein unreacted, even if all of the head protein were incorporated into prohead shells. Indeed, a large fraction of the scaffolding protein remained unreacted in the reactions analyzed in Figure 3. Reducing the scaffolding/head protein ratio to 0.33:1 only slightly reduced the yield of capsids, and even at a ratio of 0.22:1, unreacted scaffolding protein remained (Figure 4a). The yield of capsids was significantly reduced at a ratio of 0.11:1, but almost all of the head protein was depleted nonetheless. This protein was apparently converted to polycapsids consisting almost entirely of head protein, with little if any scaffolding protein, as described in more detail below.

Polycapsids are apparently large enough that they remain almost entirely in the sample well. The head protein itself barely enters the gel, and some usually remains in the sample well, so the appearance of polycapsids is not always obvious in agarose gel patterns until a considerable fraction of head protein has been used up in the reaction. A substantial fraction of the head protein appeared to be converted to polycapsids at all ratios of scaffolding/ head protein.

The approximate time course of reactions at 0.11:1 and 1:1 ratios of scaffolding to head protein was followed, initiating reactions by adding PEG200 at different times and stopping them all at once by loading into the sample wells of an agarose gel and
starting the electrophoresis (Figure 5). Material appeared at the capsid position at similar rates with either ratio of scaffolding to head protein, appearing within two minutes of incubation at 37°C, increasing substantially by 15 minutes, and increasing more slowly for the remaining two hours of reaction. Although small increases of material in the sample well were difficult to see, a significant increase was apparent by about 15 minutes of incubation and continued until most or all of the head protein was depleted, which took longer than 60 minutes. Parallel incubations of head protein alone in the presence of PEG200 did not produce any detectable capsids nor deplete the head protein (Figure 5b), indicating that the scaffolding protein is needed both for capsid formation and to catalyze conversion of head protein to polycapsids.

Fractionation of reaction products and identification of polycapsids

To purify putative capsids and polycapsids for analysis by electron microscopy, 500 µl reaction mixtures containing a constant amount of head protein with 0.33:1, 1:1 or 3:1 ratios of scaffolding/head protein were incubated along with control reaction mixtures containing scaffolding or head protein alone, all in the presence of 20% PEG200. Parallel reaction mixtures were incubated in the absence of PEG.

After two hours at 37°C, a portion of each reaction mixture was analyzed directly by agarose gel electrophoresis (Figure 6). About equal amounts of capsid were formed in the 1:1 and 3:1 reactions, with perhaps slightly less in the 0.33:1 reaction. In each case, the head protein all reacted to form capsids or polycapsids, and some scaffolding protein remained unreacted at the end of the incubation. No capsids nor apparently any polycapsids were formed in the incubations of scaffolding or head protein alone in the presence of PEG200 nor in the control reactions without PEG200.
in any of the control reactions in the absence of PEG200.

The major portion of each reaction mixture was fractionated by centrifugation through a sucrose gradient followed by agarose gel electrophoresis of a portion of each fraction to identify the components present. The results from the 1:1 reaction are shown in Figure 7. A peak of capsids is apparent near the bottom of the gradient (fractions 2 to 7) and unreacted scaffolding protein is apparent in fractions 11 to 15, the same position as purified scaffolding protein in a control gradient. As expected, no unreacted head protein is apparent near fraction 9, the position of purified head protein in a control gradient. As indicated by the presence of protein in the sample well after electrophoresis, most of the polycapsids were found in fraction 7, at the trailing edge of the peak of capsids, but smaller amounts were also apparent ahead of and trailing into the capsids (fractions 1 to 4 and perhaps 5 and 6).

Fractionation of the products of the 0.33:1 and 3:1 reactions showed similar capsid peaks near the bottom of the gradient, but the distribution of polycapsids was somewhat different (shown by Cerritelli, 1991). In the 0.33:1 reaction, polycapsids were apparent in only a single fraction just ahead of the capsids; in the 3:1 reaction, most of the polycapsids sedimented ahead of the capsids but some also seemed to be present in a single fraction just behind the capsids, about the position where most of the polycapsids from the 1:1 reaction were found. Centrifugation of the control reaction that contained head protein but no scaffolding protein showed no material sedimenting in the polycapsid region of the gradient, and denaturing gel electrophoresis indicated that the polycapsid fractions contained mostly head protein and little if any scaffolding protein (shown by Cerritelli, 1991).

Electron microscopy of negatively stained specimens from the capsid fractions from all three reactions showed the typical prohead shell structure (Figure 8, compared with Figure 2). However, differences were apparent among unstained (and unshadowed) specimens analyzed by scanning transmission electron microscopy (STEM). Most of the structures from the 3:1 reaction remained spherical, similar to the 9-10 heads isolated after co-expression of scaffolding and head protein in the same cell (Figure 9a and b). However, some of the structures from the 3:1 reaction and all of them from the 0.33:1 and 1:1 reactions had a flattened appearance with one or more splits, which we refer to as pac-men (Figure 9b and c, and not shown). Since the capsids from all three reactions looked spherical in negative stain, it seems that the capsids made at lower scaffolding/head protein ratios are less able to withstand the forces that flatten them on the grid in the absence of the supporting stain. Both
the 9-10 heads and the capsids made in the 3:1 reaction migrated slightly faster on agarose gel electrophoresis than did the capsids made in the 0.33:1 and 1:1 reactions (not shown).

Electron microscopy also confirmed the presence of polycapsids in fractions where protein remained in the sample well after electrophoresis, whether sedimenting ahead of or behind the capsids (Figure 9). The polycapsids were variable-length, tube-like structures about 50 nm wide that appeared similar to polycapsids observed after infection with amber mutants in genes 8, 14, 15 or 16 (Roeder & Sadowski, 1977; Serwer, 1979; Steven et al., 1983). As expected from their sedimentation behavior, the polycapsids that sedimented behind the capsids were considerably shorter, on average, than those that sedimented ahead of the capsids.

**Mass distribution of unstained capsids**

STEM analysis can determine the mass of unstained particles (Wall & Hainfeld, 1986), and the masses of 58 of the 9-10 heads isolated after co-expression of scaffolding and head protein in the same cell were determined, as were the masses of 93 spherical capsids and 43 flattened pac-men from the reaction at 3:1 ratio of scaffolding/head protein (Figure 10). The 9-10 heads had an average mass of 20.6 MDa, with a standard deviation of about 0.6 MDa, and the spherical capsids from the 3:1 reaction had an average mass of 20.1 MDa, with a standard deviation of about 0.9 MDa. These values are in excellent agreement with the mass of 20.2 MDa predicted for a closed shell of 420 head proteins (10% gp10B) and 140 scaffolding proteins expected from structural considerations (Dunn & Studier, 1983; Steven & Trus, 1986). The masses of pac-men were more broadly distributed toward the lower masses (Figure 10c), consistent with the idea that they may represent less completely formed capsids.

**Attempt to assemble capsids containing connector protein**

The failure to isolate connector-containing capsids from expression of the connector, scaffolding and head proteins in the same cell made it unlikely that we would obtain such capsids from the purified proteins. Nevertheless, we made an attempt to assemble them from the purified proteins under reaction conditions where capsids would form from purified scaffolding and head proteins. Assembled connectors were used, which were obtained after expression of cloned gene 8 in *E. coli* and purification through the step of hydroxylapatite column chromatography (DPH connectors, Cerritelli & Studier, 1996). Only slight differences from reactions without connectors were observed, which are summarized.

The added connectors did not eliminate the need for PEG200 in the reaction of scaffolding and head proteins to produce capsids. Reactions were tried in the absence of polyethylene glycol with head protein at 0.5 mg/ml, scaffolding protein at 0.33:1 molar ratio, and connectors at a concentration of 25 or 125 μg/ml, corresponding to approximately one capsid-equivalent of scaffolding protein and one or five capsid-equivalents of connectors, if all of the head protein were to form capsids. In 2.5 hour reactions, most of the protein remained unreacted, as analyzed by agarose gel electrophoresis, but a faint band was apparent at the position of 9-10 heads.

The same reactions in the presence of 20% PEG200 produced the usual yields of material at the positions of 9-10 heads and polycapsids, but in addition a very faint band at the position of
**Discussion**

T7 scaffolding and head protein appear both to be needed for the assembly of any capsid-like material either *in vivo* or *in vitro*. Scaffolding protein by itself seems to be a highly soluble asymmetric monomer that shows no tendency to form higher-order structures that could be detected by gel filtration, centrifugation, or agarose gel electrophoresis. Head protein by itself appears to be a tetramer in solution. The highly negatively charged scaffolding protein migrates far into the gel upon agarose gel electrophoresis at pH 8.8, whereas the nearly neutral head protein moves only slightly in the same direction. An increase in mobility of head protein with increasing amounts of scaffolding protein in mixtures (Figure 6) suggests a weak, reversible interaction between the two proteins. However, significant irreversible reaction between the purified proteins to form capsid-related structures was observed only in the presence of polyethylene glycol or dextran.

Most of our reactions used 20% PEG200, a mixture of eight and ten-carbon linear molecules of the form CH$_2$OH(CH$_2$CH$_2$O)$_n$CH$_3$. Polyethylene glycols of longer chain length were effective at lower concentrations, suggesting an excluded volume effect. Curiously, sucrose, a 12-carbon cyclic compound with eight OH groups was not effective in stimulating this reaction, although dextran T-10, a polymer of glucose, did stimulate the formation of some capsids.

The apparent formation of capsids upon co-expression of the scaffolding and head proteins in the same cell may have been triggered by a much higher effective concentration of the reactants, or perhaps by interaction with some cellular components. We did not find evidence for an effector in extracts. Rather, our results raise the possibility that most or all of the capsids isolated after co-expression of scaffolding and head protein may have been formed in the polyethylene glycol precipitation step during purification.

Capsids all appeared to be spherical shells when examined as negatively stained specimens in the electron microscope, but some collapsed into pac-men when examined without staining. Almost all of the capsids isolated after co-expression of scaffolding and head protein in the same cell, and most of those made at a 3:1 molar ratio of scaffolding/head protein, remained spherical in unstained specimens, but the capsids made at ratios of 0.33:1 or 1:1 almost all collapsed into pac-men. The capsid preparations that remained spherical also migrated slightly faster upon agarose gel electrophoresis. The differences in mobility and stability seem unlikely to be due to a difference in the amount of scaffolding protein accommodated in the protein shell, since a reaction ratio of 1:1 is already a threefold excess over the ratio of scaffolding protein found in normal prohead shells. Perhaps more likely is that closure of the capsid shell is difficult and was driven to completion in a

**Figure 10.** Mass distribution of capsids, as determined by STEM analysis. a, 58 different 9-10 heads isolated after co-expression of scaffolding and head proteins in the same cell; b, 93 spherical particles; and c, 43 pac-men from capsids produced by reaction of purified scaffolding and head proteins in a ratio of 3:1. The particles analyzed in b and c were from the same electron microscope grid.

converted heads (10-heads), a band not observed in a parallel reaction without connectors. The converted capsids could not be detected after fractionation by sucrose-gradient centrifugation, but the prohead-like capsids were isolated and analyzed further. They appeared to contain a small amount of connector protein, as analyzed by gel electrophoresis in the presence of sodium dodecyl sulfate. Analysis of negatively stained specimens by STEM showed most particles to be similar to those obtained with scaffolding and head proteins alone, but a small fraction of particles appeared to contain one, two, or even three possible connectors. Analysis of unstained specimens revealed mostly pac-men and a few uncollapsed spherical structures whereas a control reaction without connectors done at the same time showed only pac-men, suggesting that perhaps a few capsids incorporated connectors and became more stable.
significant fraction of particles only at a high concentration of scaffolding protein. If so, the spherical capsids in unstained specimens would represent completely closed shells and pac-men would arise from incomplete shells, which might be expected to flatten and break more easily under the forces generated in preparing unstained specimens, perhaps losing some subunits in the process. The mass distribution of pac-men is consistent with this interpretation, ranging from about the same mass as for spherical particles to considerably lower values (Figure 10). The absence of one pentamer or one hexamer from a closed icosahedral shell would reduce its expected mass of about 20.2 MDa by an insignificant 0.2 to 0.3 MDa, but the absence of one pentamer and five surrounding hexamers would reduce the mass by about 1.7 MDa, into the lower range of masses found for pac-men.

Polycapsids were also produced in all reactions that produced capsids. Although scaffolding protein is required for any polycapsid formation, the tubular polycapsids contained little if any scaffolding protein and seemed to have a shell structure more like converted heads than proheads. A small amount of scaffolding protein could catalyze conversion of a considerable excess of head protein into polycapsids: typically all of the head protein would be used up in a reaction and unreacted scaffolding protein would remain. Although most of the head protein usually ended up in polycapsids, polycapsid formation seemed to lag behind capsid formation (Figure 5). These results are consistent with the idea that unclosed capsids are precursors of polycapsids. It seems reasonable that polycapsids might grow from the edges of the unclosed shells, perhaps by addition of scaffolding–head protein complexes followed by a transformation that releases scaffolding protein, or perhaps by direct incorporation of head protein at the growing edge.

If unclosed capsid shells initiate the formation of polycapsids, the finding that unclosed capsids (pac-men) remained after all of the head protein had reacted implies that extension of existing polycapsids proceeded more readily than initiation of new ones, or possibly that different types of unclosed capsids were formed, only some of which could initiate polycapsids. The polycapsids produced at the 1:1 ratio of scaffolding/head protein were shorter than those produced at the 0.33:1 ratio, indicating that initiation was more efficient at the higher concentration of scaffolding protein. At the still higher ratio of 3:1, a considerable fraction of the capsid shells became closed in the reaction, thereby reducing the number of unclosed capsids available to initiate polycapsids and again producing longer polycapsids.

Purified connectors neither stimulated capsid formation nor inhibited polycapsid formation and were only poorly incorporated into capsids in the reactions we studied. Surprisingly, the capsids isolated after co-expression of connector, scaffolding and head protein had all converted to the mature capsid morphology and contained no scaffolding or connector protein. Apparently, a transient interaction of connectors and prohead shells somehow effected this conversion.

How do our results relate to the natural pathway of assembly of T7 proheads during infection? The capsid shells produced from purified or co-expressed proteins are very similar to those of natural proheads, but a substantial fraction of the purified head protein was incorporated into polycapsids in our reactions. Few if any polycapsids appear to form during wild-type T7 infection, and our reactions would be more comparable to mutants defective in genes 8, 14, 15, or 16, which specify the coaxial connector–core structure of T7, all of which give rise to both polycapsids and prohead-like capsids during infection (Roeder & Sadowski, 1977; Serwer, 1979). Apparently, the connector–core complex is not essential for capsid formation, but it somehow promotes the assembly of proheads in preference to polycapsids.

How does the connector–core complex act in the prohead assembly pathway? One possibility would be that the connector–core complex normally nucleates the formation of the prohead shell, and that this nucleation is much more rapid than the reaction of the scaffolding and head protein themselves to form unclosed shells and polycapsids. Additional assumptions would be needed to distinguish the two mechanisms of nucleating capsid formation and to explain why shells nucleated by the connector–core complex close readily whereas those nucleated in its absence appear to close only with difficulty.

A model that seems to fit the data more easily is one in which shell formation is nucleated by only one mechanism, without any involvement of the connector–core complex, and the immediate product of the reaction is incomplete prohead shells, the pac-men we observed. During normal T7 infection, these incomplete shells would be rapidly closed by insertion of the connector–core complex to form proheads, before they could react with additional scaffolding and head protein to form polycapsids or closed prohead shells. In the absence of functional connector–core complexes, such as in our reactions or during infection by some mutants, insertion could not occur, and further reaction of the incomplete shells would produce closed capsids and polycapsids. Certain mutant connector–core complexes might insert more slowly than the wild-type connectors, so that the competing reactions would produce the mixtures of aberrant proheads and polycapsids isolated after infection by these mutants. Studies using purified core proteins along with the scaffolding, head and connector proteins should be able to determine whether the connector–core complex acts by nucleation of prohead shells or by insertion into them.

The connector–core insertion model provides a natural explanation for the AG particles described by Serwer et al. (1982) and Serwer & Watson (1982). These particles behaved kinetically like intermediates in prohead assembly, gave rise to particles
reactions that alter their size and shape. (and are rapidly closed by insertion of the connector proposed for T7. Perhaps unclosed capsids assemble a connector–insertion model similar to what we their results could also be interpreted in a may be controlled by an initiating interaction protein (Guo et al, 1991). The exception was pAR2810, which carries capsids were produced in the absence of connector dispensable head fiber protein), but non-uniform shapes were assembled upon co-expression of denaturant. In f29, capsids of uniform size and shape were assembled upon co-expression of connector, scaffolding and head proteins (plus a dispensable head fiber protein), but non-uniform capsids were produced in the absence of connector protein (Guo et al., 1991). Although the authors concluded that the size and shape of the f29 capsid may be controlled by an initiating interaction between the connector and scaffolding proteins, their results could also be interpreted in a connector–insertion model similar to what we propose for T7. Perhaps unclosed capsids assemble and are rapidly closed by insertion of the connector (f29 lacks a core structure similar to that of T7), but, in the absence of a functional connector, the initially formed unclosed capsids undergo slower secondary reactions that alter their size and shape.

Materials and Methods

Clones of the connector, scaffolding and head protein genes

The clone of gene 9 used for expressing scaffolding protein was pAR441 (Studier & Moffatt, 1986), and the clone of gene 10 for expressing head protein was pAR3625 (Studier et al., 1990), both under control of their natural promoter in the silent orientation in the BamHI site of pBR322. Plasmid pAR2802 has a fragment containing genes 9 and 10 along with the natural f9 and f10 promoters and the T7 transcription terminator in pBR322, and pAR2810 has a fragment containing genes 8, 9 and 10 along with the natural promoters f9 and f10 and the T7 terminator under control of the f10 promoter in pET-1, a derivative of pBR322 (Rosenberg et al., 1987).

Expression from the cloned genes

Clones of the T7 genes were usually expressed in BL21(DE3)pLysS, which carries an inducible gene for T7 RNA polymerase in the chromosome and a plasmid that supplies a low level of T7 lysozyme (Studier et al., 1990; Studier, 1991). The exception was pAR2810, which carries all three proteins and was expressed in the presence of pLysE, whose higher level of lysozyme decreased basal expression in the uninduced cell and made it easier to maintain the plasmid before induction. Cultures were grown and induced in 500 ml of M9TB plus 20 μg ampicillin/ml in one liter flasks shaking at 37°C. Induction was by 0.4 mM IPTG added when the culture reached an optical density of about 0.8 at 600 nm, and cells were collected two or three hours later by centrifugation. The T7 proteins accumulated to become a major fraction of the total cell protein.

Protein purification

Scaffolding protein was very soluble but quite susceptible to proteases. Good extracts could be made from cells suspended in 1/20 of the original volume in buffer A (20 mM Tris-HCl (pH 8.0), 3 mM β-mercaptoethanol, 4% (v/v) glycerol, 2 mM Na3EDTA) containing 20 μg/ml of the protease inhibitor z-toluene sulfonyl fluoride (PMSF, Eastman), by making the suspension 0.1% in Triton X-100 and freezing three times in a solid CO2/ethanol bath, with thawing at room temperature. The T7 lysozyme supplied by pLysS or pLysE assured thorough lysis (Studier, 1991). Head protein was largely insoluble when extracts were prepared in this way but was mostly soluble if the cells were concentrated only fivefold instead of 20-fold and 0.1 M NaCl was added to the suspension buffer. However, the head protein would slowly precipitate, so purification had to proceed rapidly. Higher NaCl concentrations seemed to retard the precipitation.

Extracts were made 10 mM in MgSO4 and treated with DNase I (10 μg/ml) for 15 minutes at room temperature. After removing cell debris by centrifugation, the proteins were purified from the supernatant by column chromatography over DEAE-Sepharose CL-6B (Pharmacia), Cellulose Phosphate P-11 (Whatman) and Bio-Gel HTP Hydroxylapatite (BioRad). Gel filtration chromatography was performed through a Superose 12 column (HR 10/30, with a bed volume of 23 ml) using the Fast Protein Liquid Chromatography (FPLC) system (Pharmacia). This column was calibrated by monitoring the elution pattern of proteins of known molecular mass (BioRad). Purification was monitored by electrophoresis through a polyacylamide gradient gel in the presence of sodium dodecyl sulfate followed by staining with Coomassie brilliant blue. The protein profiles of uninduced and induced cells, and of fractions from the column separations described below, are given by Cerretelli (1991).

Purification of the T7 scaffolding protein

A 100 ml extract of cells from two liters of culture of BL21(DE3)pLysS/pAR441 collected three hours after induction was loaded onto a 25 ml column of DEAE-Sepharose CL-6B equilibrated with buffer A, washed with 60 ml of 50 mM NH4Cl in buffer A, and eluted with a 240 ml linear gradient of 50 mM to 500 mM NH4Cl in buffer A. The peak fractions of scaffolding protein, which eluted near 0.3 M NH4Cl, were pooled, diluted twofold in buffer A to give 80 ml, and loaded onto a 15 ml hydroxylapatite column equilibrated with buffer A. After washing with 40 ml of buffer A, the column was eluted with a 200 ml gradient of 0 to 0.5 M sodium phosphate (pH 7.0) in buffer A. Most of the scaffolding protein remained in the flowthrough, with a small amount
eluting near the beginning of the gradient. A 10 ml sample of the flowthrough of the hydroxylapatite column was concentrated to 200 μl in a Centricon-30 microconcentrator (Amicon) in buffer A containing 50 mM NH₄Cl and passed through a Superose 12 column equilibrated with the same buffer. The pooled peak fractions appeared to be greater than 98% pure (Figure 1). The purified protein was stored at −80°C in small aliquots and used for the experiments described herein; once an aliquot had been thawed it was stored at 4°C and used for no longer than three days.

**Purification of the T7 head proteins**

A 200 ml extract was made from cells collected two hours after induction of one liter of BL21(DE3)pLysS/pAR3625. This extract, in buffer A containing 0.1 M NaCl, was passed through a 25 ml column of DEAE-Sepharose CL-6B equilibrated with the same buffer, to which neither the gp10A nor gp10B head protein binds. The flowthrough and wash fractions were immediately loaded onto a 15 ml hydroxylapatite column equilibrated with buffer A containing 0.1 M NaCl. After washing with 80 ml of the same buffer, the column was eluted with a 200 ml linear gradient of 0 to 0.5 M sodium phosphate (pH 7.0) in buffer A. The gp10A and gp10B bound to the column and co-eluted at approximately 0.2 M phosphate. A 2 ml sample of the pooled peak fractions (60 ml) was concentrated to 200 μl in a Centricon-30 microconcentrator (Amicon) in buffer A containing 0.5 M NaCl and passed through a Superose 12 column equilibrated with the same buffer. Again, the gp10A and gp10B proteins co-eluted. The peak fractions were stored individually at −80°C, and only those fractions that contained the purest protein, estimated to be greater than 98% gp10 (Figure 1), were used for the experiments described herein.

**Incubations to form capsids and polycapsids**

Standard reactions contained purified head protein at concentrations of 0.5 to 1 mg/ml (14 to 27 μM of monomer) and different molar ratios of scaffolding protein in 50 mM NaCl, 50 mM Tris-HCl (pH 8.2), 20% (w/v) PEG200, and were incubated at 37°C and maintained at −160°C on the liquid N₂-cooled stage. Freeze-dried specimens were prepared as described (Mosesson et al., 1981; Wall et al., 1985). A 3 μl sample, containing 50 to 100 μg protein/ml, was added to a drop of 20 mM ammonium acetate on a 20 to 30 Å thin carbon film supported by a holey thick carbon film on a titanium grid, to which an internal mass calibration standard of tobacco mosaic virus (3 μl of a 100 μg/ml stock) had been previously applied. After allowing attachment for one minute, the grids were washed 10 to 15 times by wicking with filter paper and adding 5 μl of the same buffer. Finally, excess fluid was drawn off with filter paper, and the grid frozen by rapid immersion into a liquid nitrogen/ethanol slush, and dried under vacuum for a period of six to eight hours. Negatively stained specimens were prepared in a similar fashion, except the samples were air-dried from 2% (w/v) uranyl acetate. Conventional transmission electron microscopy used a Philips 300 instrument.

Since the fraction of elastically scattered electrons in STEM is proportional to thickness, mass analysis of the image data can be performed (Wall & Hainfeld, 1986). Briefly, mass analysis programs were used to subtract background and calculate masses, using tobacco mosaic virus as an internal standard. Boundaries around particles to be measured were selected interactively.

**Gel electrophoresis**

Electrophoresis at 3 to 6 V/cm through 1.75% (w/v) agarose gel in 40 mM Tris-acetate (pH 8.8), 2 mM Na₃EDTA provided good resolution among scaffolding protein, head protein and various forms of capsids, and was the condition used to analyze reaction products unless indicated otherwise. The pH was increased to 8.8 from the usual 8.0 so that head protein would move farther into the gel; head protein itself remained mostly in the sample well when the pH of the gel buffer was as high as 8.2. Electrophoresis was usually stopped after the bromophenol blue tracking dye had moved about 8 cm from the origin, and proteins were visualized by staining in 250 ml of 10% (v/v) acetic acid to which 5 ml of 0.25% Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid was added. The gel was rocked gently at room temperature overnight and no destaining was necessary.

Purified proteins and the protein composition of capsids were analyzed by heating the sample in a boiling water bath followed by electrophoresis through a 10% to 20% polyacrylamide gradient gel, both in the presence of sodium dodecyl sulfate, and staining with Coomassie brilliant blue (Studier & Moffatt, 1986).

**Sucrose gradient fractionation**

Sucrose gradients were formed from 3.7 ml of a solution consisting of 50 mM NaCl, 50 mM Tris-HCl (pH 8.0), 2 mM Na₃EDTA, 2 mM β-mercaptoethanol and 12.5% (w/v) sucrose in a polyallomer centrifuge tube cycled four times through freezing at −20°C and thawing at room temperature (de la Campa et al., 1987). Samples were layered onto the gradients and centrifuged 35,000 rpm at room temperature for 30 minutes in a Beckman SW50.1 swinging bucket rotor. After centrifugation, the contents of the tubes were fractionated by collecting drops through a hole punched in the bottom of the tube. Usually, 18 or 19 fractions of about 0.2 ml were collected.

**Electron microscopy**

Scanning transmission electron microscopy (STEM) was performed using the Brookhaven Biotechnology Resource Instrument (Wall, 1979; Wall & Hainfeld, 1986). The operating voltage was 40 keV, and all specimens were maintained at −160°C on the liquid N₂-cooled stage. Freeze-dried specimens were prepared as described (Mosesson et al., 1981; Wall et al., 1985). A 3 μl sample, containing 50 to 100 μg protein/ml, was added to a drop of 20 mM ammonium acetate on a 20 to 30 Å thin carbon film supported by a holey thick carbon film on a titanium grid, to which an internal mass calibration standard of tobacco mosaic virus (3 μl of a 100 μg/ml stock) had been previously applied. After allowing attachment for one minute, the grids were washed 10 to 15 times by wicking with filter paper and adding 5 μl of the same buffer. Finally, excess fluid was drawn off with filter paper, and the grid frozen by rapid immersion into a liquid nitrogen/ethanol slush, and dried under vacuum for a period of six to eight hours. Negatively stained specimens were prepared in a similar fashion, except the samples were air-dried from 2% (w/v) uranyl acetate. Conventional transmission electron microscopy used a Philips 300 instrument.

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