# FUNCTIONS OF SCAFFOLDING PROTEIN IN PHAGE P22 CAPSID ASSEMBLY AND MATURATION

by

Barrie Greene

A.B., Physics
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Submitted to the Department of Biology
in partial fulfillment of the requirements for the degree of

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## ABSTRACT

While the structures of many viruses are known to high resolution, the detailed pathways by which they are assembled are not. In the case of the dsDNA bacteriophages, herpesviruses and adenoviruses, the initial product of assembly is not the mature, DNA-containing virion but a precursor particle. These procapsids contain proteins not found in the mature virion, termed scaffolding proteins, which are essential for capsid assembly. Assembly of the bacteriophage P22 procapsid requires approximately 300 molecules of a 33 kD scaffolding protein in addition to 420 coat protein subunits. During the process of DNA packaging and phage maturation all 300 scaffolding molecules exit the procapsid through channels in the coat lattice, the DNA is packaged, and the capsid undergoes conformational changes leading to expansion and closure of the channels. In this work I attempt to determine the roles played by the scaffolding subunits in these complex processes and to identify regions of the molecule required for specific functions.

Scaffolding release can be reproduced *in vitro* in the absence of DNA by low concentrations of guanidine hydrochloride. This release from within the capsid was reversible, permitting study of scaffolding interactions with the assembled coat lattice. The rapidity of scaffolding reentry suggested that the subunits could rebind to specific sites within the capsid. Procapsids contained two classes of scaffolding subunits, which may represent binding to different sites within the lattice; thus, all scaffolding proteins do not make equivalent interactions with the coat protein. These sites became lost or inaccessible upon capsid expansion and maturation. Image reconstruction from cryo-electron micrographs of procapsids containing scaffolding suggest that although the scaffolding subunits interact with the capsid lattice, the

scaffolding core is not arranged with the same icosahedral symmetry as the outer shell.

A set of new missense mutants at four sites in the scaffolding protein were isolated and characterized. These mutants did not prevent capsid assembly under restrictive conditions. Two mutants were defective in incorporation of the portal complex which serves as the channel through which DNA is packaged. These mutations may identify a region of the protein required for interaction with the portal. Two mutants in a different region of the sequence were impaired in scaffolding release both *in vivo* and *in vitro*. These mutations may identify a new domain required for scaffolding release. Both these mutations resulted in severe destabilization of part of the protein to thermal denaturation. Scaffolding release appeared to be required for capsid expansion; in turn, scaffolding release seemed to depend upon the presence of a portal. This may help to order the pathway of events in phage maturation.

A scaffolding region required for binding to coat protein was identified by functional assays of proteolytic fragments, and found to be the C-terminal 20-30 residues. The structural organization of the purified wild-type and mutant proteins were also probed by protein denaturation techniques. The unfolding of scaffolding protein was not a simple two-state mechanism as for a typical globular protein, but a complex process invoving the sequential denaturation of multiple domains. The mutant amino acid substitutions selectively destabilized particular domains, allowing them to be assigned to known functions. The scaffolding protein was notably unstable, to the extent that some domains are probably largely unstructured at physiological temperatures. The less stable domains include the regions involved in coat binding, portal insertion and scaffolding release, suggesting that many critical scaffolding functions may require a high degree of conformational flexibility.

Based on these results I propose a model for assembly in which a terminal region of the scaffolding protein induces conformational changes in the coat protein leading to efficient polymerization. The release of scaffolding may not be a passive result of changes within the coat lattice but involve active conformational changes within the scaffolding subunits in response to signals associated with DNA packaging. Docking of the DNA packaging complex at the portal could cause a signal to be propagated throughout the scaffolding core resulting in scaffolding release, after which the coat lattice is freed to expand to its mature conformation.

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TABLE OF CONTENTS

LIST OF FIGURES

## LIST OF TABLES

# CHAPTER 1

# INTRODUCTION:
# VIRAL SCAFFOLDING PROTEINS

## ASSEMBLY OF VIRUSES WITH SCAFFOLDING PROTEINS

The double-stranded DNA within a virus capsid is condensed over 200-fold with respect to that free in a cell (Earnshaw and Harrison, 1977). Double-stranded DNA, however, is not easily compressible (Gray and Hearst, 1968). While RNA viruses are believed to assemble by coconsdensation of capsid proteins around their nucleic acid (Sorger et al., 1986; Schneemann et al., 1994), this mechanism is not possible for viruses containing dsDNA. Some dsDNA viruses, such as SV40, use cell histones to condense their DNA into a compact mini-chromosome (Griffith, 1975), which can then be covered by capsid proteins. But other dsDNA viruses, including herpesviruses, adenoviruses, and the dsDNA bacteriophages, adopt a different strategy. They build the outer protein capsid first, then actively pump the DNA into it.

Thus the first product of the assembly pathway for these viruses is is not the mature virion, but a precursor capsid empty of DNA. In place of the DNA, several hundred molecules of a protein or proteins fill the interior of the procapsid. These are required for assembly of the procapsid, but not found in the mature virion. These proteins have been termed "scaffolding" proteins (King and Casjens, 1974; Casjens and Hendrix, 1988).

### The assembly pathway of dsDNA bacteriophage

The overall pathway of dsDNA bacteriophage assembly (reviewed in Hendrix, 1985; Casjens and Hendrix, 1988) has been well characterized and

shares common features in all the dsDNA phages studied. Assembly of a phage procapsid (see Fig. 1.1) involves three essential proteins: the major capsid, or coat protein; the scaffolding; and a portal protein which makes up a dodecameric ring complex at one icosahedral vertex that serves as the channel through which the DNA is packaged (Bazinet and King, 1985). Other minor capsid components may also be included. While the procapsid contains the same number of coat subunits as the mature virion, the appearance of the procapsid is different, having a smaller size, thicker, rougher edges, and a rounded rather than icosahedral shape.

During the transition of the procapsid to the mature virion, the scaffolding molecules are removed from the procapsid, either by proteolytic cleavage, as in the case of T4 or lambda (Onorato and Showe, 1975; Ray and Murialdo, 1975), or by exiting intact to be recycled into new procapsids, as for P22 and phi29 (King and Casjens, 1974; Nelson et al., 1976). The DNA is pumped into the procapsid in a process that requires phage-encoded packaging proteins and ATP (Black, 1989). The capsid undergoes structural alterations that result in a more regular icosahedral shape and an expansion of the capsid volume (Fig. 1.2). Capsid proteins may be proteolytically processed, and external proteins may be added. The DNA is stabilized by the addition of proteins at the portal vertex, and the phage tail is attached.

The assembly pathways of both herpesviruses (Sherman and Bachenheimer, 1988, Lee et al., 1988) and adenoviruses (D'Halluin et al., 1978; Edvardsson et al., 1976) also include precursor capsids containing proteins not found in the mature virion and believed to be the equivalent of phage scaffolding proteins (Rixon, 1993; Hasson et al., 1992).

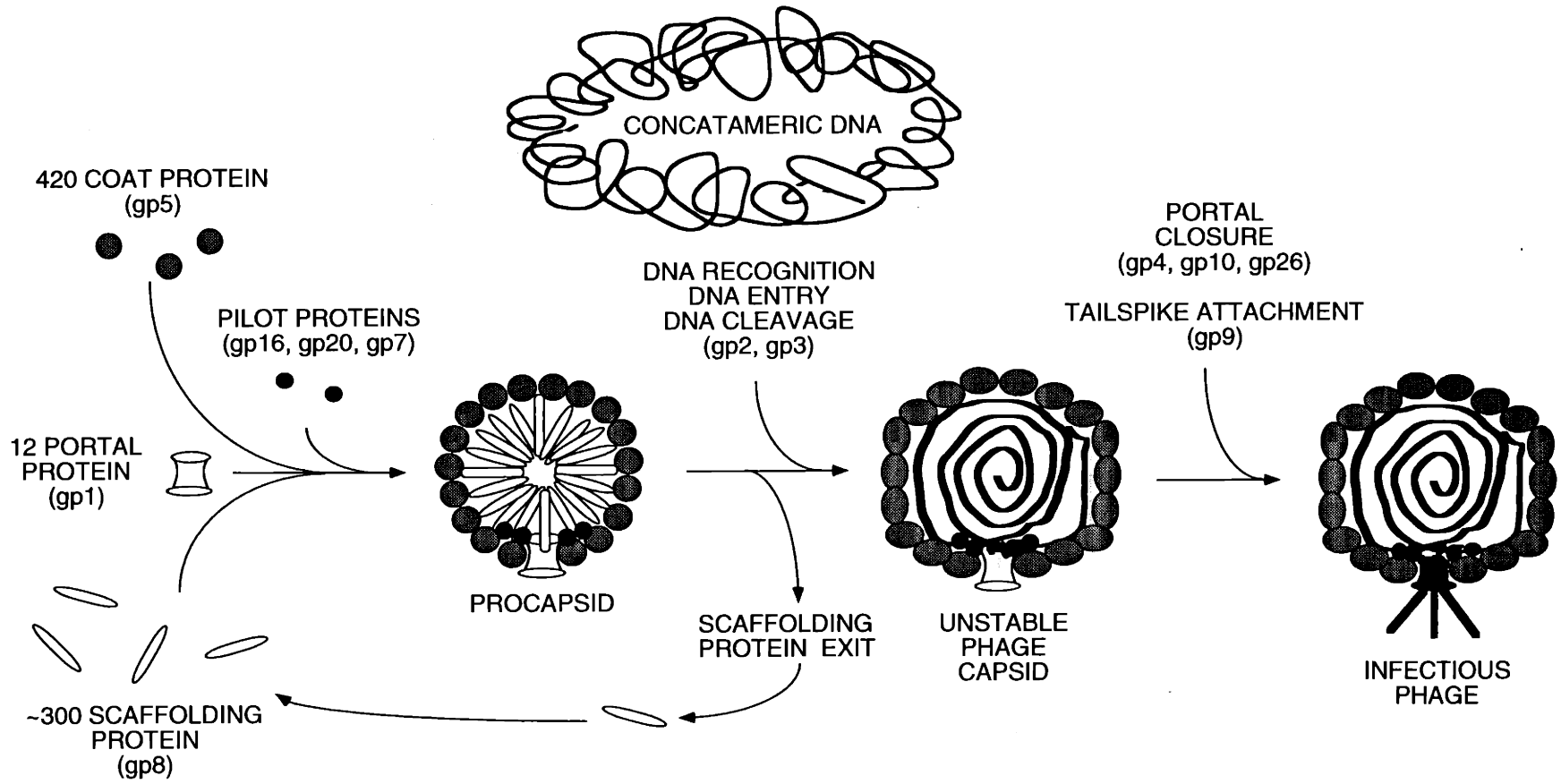
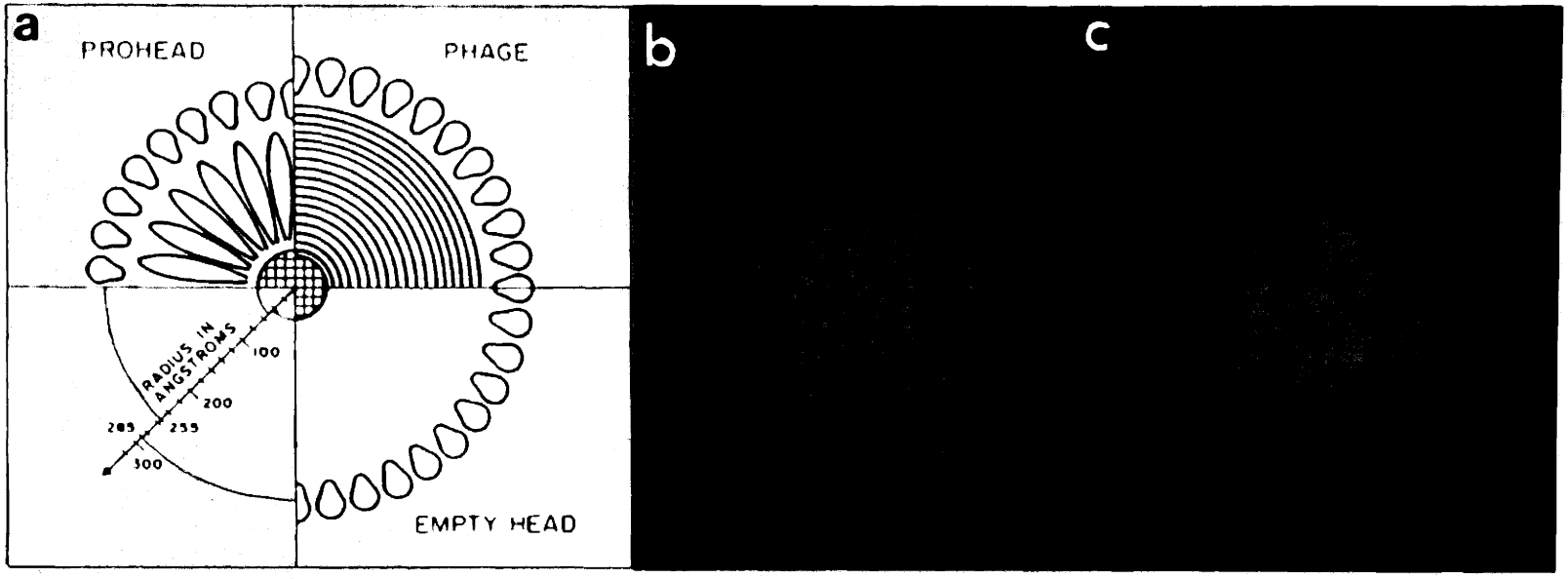



Figure 1.1 Assembly pathway of bacteriophage P22

FIG. 1.2 Structure of the P22 procapsid. a) structure of the procapsid (prohead), mature phage, and empty mature phage (empty head) as determined by x-ray scattering (Earnshaw et al., 1976). Cryo-EM image reconstruction of b) extracted procapsid shell, and c) phage (Prasad et al., 1993).


a
PROHEAD
PHAGE
RADIUS IN ANGSTROMS
100
200
255
285
300
EMPTY HEAD
b
c

**Functions of scaffolding proteins in assembly**

What is the role of the scaffolding proteins? Some element of the assembly pathway for these viruses must dictate the need for large numbers of a protein only transiently associated with a morphogenetic intermediate. Several functions for these proteins have been proposed:

— Morphogenesis. As the term "scaffolding" implies, these proteins may assist the major capsid subunits in the tricky problem of forming a topologically closed empty shell. In the absence of a size-determining core of nucleic acid, it is not clear how capsid proteins would "know" how to assemble a capsid of the correct size, since the proteins at opposite edges of a growing shell cannot communicate with each other. The scaffolding molecules could provide a form-determining core, or help join capsid subunits to each other with the correct curvature (Hendrix, 1985; Kellenberger, 1990). The scaffolding would then be removed to make room for the DNA.

— Shielding of the coat protein from incorrect associations. There are other potential problems involved with the assembly of an unfilled shell. The viral DNA might interact with the inner surface of a partially formed shell before it has closed, thus hindering correct assembly (King and Chiu, 1995). Since the virions assemble within the crowded interior of the host cell, it might be possible for extraneous proteins to become trapped within the growing shell as it closes, thus blocking the entry of DNA (Earnshaw and Casjens, 1980). By binding to the inside of the assembling shell, the scaffolding subunits could prevent the premature interaction with DNA or the incorporation of proteins for which the virus has not evolved a removal mechanism.

— Incorporation of a DNA packaging portal. The viral DNA must be provided some means of entry into the preformed shell. How this occurs in

the animal viruses is not known. The bacteriophages contain a structure at one of the five-fold vertices termed a connector (since it serves to connect the phage head to the tail) or portal (Bazinet and King, 1985). This structure is composed of 11, 12 or 13 molecules of a portal protein arranged in a ring, and is believed to be the channel through which DNA enters the phage head. Since the portal complex sits at a five-fold vertex there is a symmetry mismatch (Moody, 1965). Hendrix (1978) proposed that the portal may rotate during DNA packaging. The scaffolding protein may be needed for the incorporation of such a portal complex into the unique capsid vertex.
— DNA packaging. Since the scaffolding protein occupies the interior of the capsid where the DNA will go, it is possible that it might help to place or stabilize the initial DNA into the capsid before exiting. Alternatively, scaffolding release might be energetically coupled to DNA entry (King and Casjens, 1974).

In this introduction I will consider the properties and functions of this intruiging class of proteins. The availability of nonsense mutations in key structural proteins and the ease of purifying large quantities of structures from infected cells has long made bacteriophage the system of choice for studying morphogenesis. Many features of the phage assembly pathway are only now being appreciated for the animal viruses. The properties of the phage scaffolding proteins and their roles in assembly will first be considered for the well characterized dsDNA phages. Evidence that two classes of animal viruses possess proteins with analogous functions will then be discussed.

## THEORY OF VIRUS STRUCTURE

Crick and Watson (1956) were the first to point out that since viruses have little coding capacity, they should be highly symmetric in structure. In this way a single virally encoded protein can repeatedly make identical sets of contacts so as to build the entire viral capsid. Viruses may have either helical symmetry (as for Tobacco Mosaic Virus and others) or cubic symmetry, as for all the viruses discussed here. In fact, all non-helical viruses are icosahedral in shape. An icosahedral surface can accommodate the largest possible number of identical subunits; 60 such subunits can fit on the surface of an icosahedron, 3 on each of 20 triangular faces, with each one making equivalent 2-fold, 3-fold and 5-fold contacts with its neighbors.

It was soon realized, however, that most icosahedral viruses contained more than 60 subunits. Caspar and Klug (1962) suggested that this difficulty could be resolved by subtriangulating the faces of the icosahedron. Rather than 60 identical subunits, the larger icosahedron will contain 60 identical asymmetric units, each containing T subunits, where T is one of a set of integers satisfying the condition $T = h^2 + hk + k^2$. The different subunits within the asymmetric unit no longer make exactly the same set of contacts. For example, those subunits at icosahedral vertices make a five-fold contact with their neighbors, while other subunits make a six-fold contact. The differences in bonding angles between the different positions, however, should not be large. According to Caspar and Klug, the subunits are now "quasi-equivalent," in that only slight deformations of the protein fold are needed to accommodate the new interactions.

While some violations of quasi-equivalence have been observed — most notably in the polyoma viruses, where both pentavalent and hexavalent positions are occupied by pentamers (Rayment et al., 1982) — for the most

part virus capsids do demonstrate the expected number of quasi-equivalent conformations. In contrast to Caspar and Klug's predictions, atomic resolution structures of viruses have proven that the quasi-equivalent conformers do not represent slightly bent forms of the same fold. Instead, the bulk of the subunit has an identical conformation in all positions, while small extended "arms" that mediate contacts between subunits adopt markedly different structures (Rossmann and Johnson, 1989; Liddington et al., 1991).

The differences in the quasi-equivalent phage subunits appear to be more global. The structure of the P22 procapsid (Fig. 1.2b) demonstrates that the quasi-equivalent subunits vary in shape. Since changes in small arms would not be visible at this resolution, the differences in these subunits may result from alternate conformations of a hinge region.

Thus the problem of virus morphogenesis is how to determine the correct T number, which requires switching specific regions in identical protein subunits so that they can make non-identical contacts. The crystal structures of some RNA viruses revealed ordered RNA at key points in the structure, interacting with the arms of the C subunits so as to shift them into a conformation different from those of the A and B subunits and promote the assembly of a T=3 capsid (Fisher and Johnson, 1992; Wery et al., 1993). In the case of SV40, the condensed DNA is thought to serve as a template that constrains the curvature of the growing shell. (Liddington et al., 1991). Presumably some comparable role is filled in part by the scaffolding proteins in those viruses which contain them.

## PROPERTIES OF SCAFFOLDING PROTEINS

While the structures of many viruses have been solved to atomic resolution, none of these structures have included scaffolding. No purified scaffolding protein from any virus has yet been successfully crystallized. Nor do the sequences of these proteins indicate homology to any proteins of known structure. Thus, little is known about the three-dimensional structures of these molecules.

The sequences of the scaffolding proteins from a number of phages (T4, P22, T7, T3, Phi29, lambda, P2) have been determined (Marusich and Mesyanzhinov, 1989; Eppler et al., 1991; Dunn and Studier, 1983; Vlcek and Paces, 1986; Sanger et al., 1982 ; Linderoth et al., 1991). No sequence homology is apparent, except between closely related phages such as T7 and T3. This does not rule out the possibility of structural homology, as the coat proteins of the icosahedral RNA viruses studied to date, while also displaying no detectable sequence homology, have proven to possess very similar beta barrel structures (Rossmann and Johnson, 1989). The phage portal proteins, while also non-homologous on the primary structure level, all assemble into strikingly similar dodecameric channel complexes (Bazinet and King, 1985).

The scaffolding proteins of different phages (Table 1.1) do have several properties in common. All are predicted to be highly alpha-helical based on their sequences (Eppler et al., 1991). These predictions have been verified for the scaffolding protein of P22, for which both Raman spectroscopy (Thomas et al., 1982) and circular dichroism (Teschke et al., 1993) demonstrate substantial alpha-helical structure. The sequences of the scaffolding proteins of phages phi29, lambda, and T7, as well as of herpesvirus, contained a leucine zipper motif (Guo et al., 1991). This motif may mediate dimerization between

scaffolding subunits, as it does for several DNA-binding proteins (Landschulz et al., 1988).

The shapes of the T4, P22 and lambda scaffolding proteins have been characterized by velocity sedimentation or gel filtration (van Driel, 1980; Fuller and King, 1982; Ziegelhoffer et al., 1992). All appear to be highly elongated molecules, with calculated axial ratios of 20, 9 and 13 respectively. The scaffolding proteins vary in size, however. The scaffolding protein of P22, at 33.6 kD, is over twice the size of the lambda scaffolding protein, although both assemble into T=7 procapsids.

Many of the scaffolding proteins have a high proportion of charged residues (Eppler et al., 1991). In some cases the distribution of charges is distinctly non-random, with acidic amino acid residues concentrated at the amino-terminal ends and basic residues at the carboxy-terminal ends. These charges may be involved in electrostatic interactions during assembly.

Casjens and Hendrix (1988) have noted that all the dsDNA phages sequenced so far have a similar gene order. There is some evidence that genes encoding proteins which interact are near one another in the DNA sequence. The lambda genes Nu1, A and B, encoding the DNA packaging proteins and the portal, lie consecutively along the lambda sequence (Sanger et al., 1982). During DNA packaging, the C-terminus of gpNu1 interacts with gpA, while the N-terminus of gpA interacts with gpNu1 and the C-terminus of gpA interacts with the procapsid, probably binding to the portal protein gpB (Feiss, 1986; Wu et al., 1988). The gene encoding the scaffolding protein has a conserved position between the portal and coat genes, perhaps an indication that the N and C-termini of the scaffolding interact with portal and coat, respectively.

TABLE 1.1

Scaffolding proteins of bacteriophage and animal viruses

| Virus | scaffolding protein | molecular weight | approx. # per capsid | capsid size | references |
|---|---|---|---|---|---|
| P22 | gp8 | 33.6 kD | 300 | T=7 | Eppler et al., 1991<br>Casjens, 1979, Prasad et al., 1993 |
| T4 | gp22 | 29.9 kD | 580 | T=13 (prolate) | Marusich and Mesyanzhinov, 1990; Black et al., 1994 |
| | gp67 | 8.9 kD | 340 | | Keller et al., 1986 |
| | gp68 | 15.7 kD | 240 | | Volker et al., 1982 |
| lambda | gpNu3 | 13.4 kD | 200 | T=7 | Dunn and Studier<br>Dokland and Murialdo, 1993 |
| phi29 | gp7 | 11.3 kD | 180 | T=3 (prolate) | Anderson and Reilly, 1993 |
| T7 | gp9 | 34 kD | 200 | T=7 | Roeder and Sadowski, 1977 |
| P2 | gpO | 31.4 kD | 95 | T=7 | Lindqvist et al., 1993 |
| HSV-1 | VP22 | 40 kD | 1100 | T=16 | Newcomb and Brown, 1991<br>Robson and Gibson, 1989 |
| EHV | VP22 | 46kD | 600 | T=16 | Newcomb et al., 1989 |
| Adenovirus | L152-55 kD | | 100+ | T=25 | Hasson et al., 1989; 1992 |

The gene encoding the lambda scaffolding protein, Nu3, is located within the coding region of gene C, whose product is a component of the lambda connector (Shaw and Murialdo, 1980). The sequence of gpNu3 is thus identical with the C-terminal third of gpC. As the lambda genome is sufficiently large not to require such overlap, this arrangement may have functional significance. The common sequence may allow for interactions between the two proteins during assembly.

## FUNCTIONS OF THE BACTERIOPHAGE SCAFFOLDING PROTEINS

### Morphogenesis

*Isometric phage*

The isometric coliphages T7 and lambda, and the *Salmonella* phage P22, normally produce T=7 capsids. Elimination of scaffolding protein by nonsense mutations results in the accumulation of many "spirals," large structures of varying curvature which fail to close (King et al., 1973; Roeder and Sadowski, 1977; Ray and Murialdo, 1975). The overall yield of structures is reduced, with higher concentrations of coat protein within the cell required before it will associate. The P22 coat protein also produces some small capsids, probably T=4 in size (Earnshaw and King, 1978).

Some normally sized capsids are produced in these infections, indicating that the coat protein contains within itself the necessary information to determine the capsid curvature, but the presence of scaffolding greatly increases the efficiency of correct polymerization. The scaffolding protein is sufficient to insure the construction of properly sized shells for the isometric phages, as amber mutations in other proteins, including the portal, have no effect on morphogenesis (Serwer and Watson, 1982; King et al, 1973; Ray and Murialdo, 1975). Procapsids of P22 can be

assembled *in vitro* from purifed scaffolding and coat subunits alone (Fuller and King, 1982; Prevelige et al., 1988).

The bacteriophage P2 and its satellite phage P4 provide a particularly interesting example of size regulation (Lindqvist et al., 1993). P2 normally forms T=7 capsids of gpN, with the assistance of the scaffolding protein gpO (Lengyel et al., 1973). In coinfected cells, P4 uses the P2 coat protein, gpN, to form T=4 shells, too small to package the larger P2 genome (Dokland et al., 1992). The production of these smaller shells is controlled by the product of the P4 sid gene (Barrett et al., 1976), which is found only in P4 procapsids. When gpN is expressed from a plasmid, it can form either size shell depending upon which of the two scaffolding proteins is present (Marvik et al., 1994). The sid protein was recently found to form an external scaffolding around the T=4 procapsids (T. Dokland and B. Lindqvist, unpublished results). Production of viable P4 phage also requires gp0 (Six, 1975), suggesting that this internal scaffolding has functions in addition to morphogenesis.

*Prolate phage*

Assembly of the prolate phages is a more complex problem, as these viruses have shapes based on icosahedral latttices, but with extra bands of capsomeres inserted between the ends of the shell so as to produce an elongated structure; thus these phage must regulate both the size and the length of their capsids. Their assembly also requires scaffolding proteins. Phi29, a phage of *Bacillus subtilus*, forms an extended T=3 structure (Anderson and Reilly, 1993). In the absence of its scaffolding protein, spiral-like particles are produced (Hagen et al., 1976). The coliphage T4 has a much larger extended T=13 capsid. Assembly of T4 (Black et al., 1994) is a complex process requiring both the major capsid protein gp23 and a separate protein,

gp24, that forms the fivefold vertices. In addition to the major scaffolding protein gp22, the T4 procapsid includes the minor scaffoldings gp67 and gp68, as well as the dispensible core proteins IP I-III. Mutations in the major scaffolding protein, gp22, result in the formation of multilayered polytubes, long tubes of coat protein (Laemmli et al., 1970). These are presumed to arise from a defect in the ability to correctly initiate procapsid assembly. The polytubes represent aberrant initiation events, but are also defective in the termination or capping steps.

While scaffolding protein is necessary, it is not sufficient to determine the shapes of prolate phage. Cells infected with amber mutants of the phi29 portal protein form only isometric particles (Hagen et al., 1976). Expression of the coat and scaffolding proteins in *E.coli* resulted in particles ranging in length from isometric to the normal prolate size. Coexpression of the portal protein was required to produce uniformly prolate procapsids (Guo et al., 1991).

Amber mutations in the T4 portal protein resulted in formation of polyheads (Laemmli et al., 1970). While these polyheads had only a single layer, and were closer in diameter to the normal procapsid (Steven et al.,1976), they did not have closed ends. Reconstitution of T4 procapsids *in vitro* from coat and core proteins yielded only polytubes (van Driel, 1977; Caldentey et al., 1987). Addition of a complex including the portal protein is required to form procapsids of normal length (van Driel and Couture, 1978a).

Mutations in either the major T4 scaffolding, gp22 (Paulson et al., 1976; Keller et al.,1988), or the additional scaffolding proteins, gp67 and gp68 (Volker et al., 1982a; Keller et al., 1986, 1988), cause assembly of many biprolate or wider than normal phage. Although mutations in several proteins can result in phage of varied lengths, only mutations in the scaffolding proteins

alter capsid width (Black et al., 1994) leading to the proposal that scaffolding is essential for regulation of this dimension (Keller et al., 1988). The scaffolding proteins appear to be sufficient to determine the width, or the T number, of a viral capsid, while additional information from the portal is required to specify length.

**Mechanism of assembly**

Several models have been proposed for how the scaffolding molecules could direct the polymerization of the coat protein. One possibility is that the scaffolding subunits form a core around which the coat molecules assemble. The core model appears unlikely, as no preformed scaffolding cores have been detected for isometric phages, although both lysates and thin sections of cells infected with coat mutants have been examined. (King et al., 1973; Lenk et al., 1976; Zachary et al., 1976). A mutation in the coat protein of lambda results in the formation of small T=4 capsids (Katsura, 1983). These capsids contain a small scaffolding core, although the scaffolding protein is wild-type. That a mutant coat protein can direct the scaffolding into an alternatively sized core argues against the existence of preformed cores.

Naked cores containing the T4 scaffolding and core proteins have been observed and purified (Traub and Maeder, 1984; Traub et al., 1984), and pulse-chase experiments suggested that these cores could be covered with coat protein *in vivo* after a temperature shift (Kuhn et al., 1987). However, there is no evidence that these cores are normal assembly intermediates. The fact that mutations in the T4 coat protein cause assembly of polytubes containing elongated cores (Doermann et al., 1973) implies that the T4 core normally coassembles with the coat protein.

The procapsid assembly process has been studied in greatest detail for bacteriophage P22, since it has been shown that purified coat and scaffolding subunits can be efficiently assembled *in vitro* in the absence of any other factors (Fuller and King, 1982; Prevelige et al., 1988). Under the same conditions, the scaffolding protein by itself does not assemble into any large structures. In experiments where scaffolding protein was limiting, a minimum of about half the usual number of scaffolding molecules per capsid were found to be required for assembly (Prevelige et al., 1988), arguing against models in which the scaffolding is only required for initiation. When assembly intermediates were trapped by crosslinking and examined in the electron microscope, partial double layered shells were observed (Prevelige et al., 1993b). This suggests that both coat and scaffolding proteins add on to the edges of an assembling shell. The proteins probably add as monomers, since no small heteroligomers have been observed for P22 or other phage proteins (Prevelige et al., 1988; 1993; Hendrix and Casjens, 1988).

**Structure of the scaffolding core**

For the isometric phages P22 and lambda, small-angle x-ray scattering data support models in which the scaffolding protein is arranged in a central spherical core within the outer shell of coat protein (Earnshaw et al., 1976; 1979). Structures of procapsids from P22 and lambda have been solved by cryo-electron microscopy and image reconstruction to 25-35 Å resolution (Prasad et al., 1993; Dokland and Murialdo, 1993). The arrangement of scaffolding protein within these shells has not yet been determined however, since these procapsids had had their scaffolding removed by extraction *in vitro* (P22) or proteolysis *in vivo* (lambda). It is not yet known whether or not the

scaffolding is even arranged within the procapsid with icosahedral symmetry matching that of the outer shell.

The structure of the core within T4 polyheads has been analysed by electron microscopy and optical diffraction of electron micrographs (Paulson and Laemmli, 1977; Engel et al., 1982). The core structure appears to be a 6-fold set of gp22 helical chains, similar to those made by gp22 *in vitro* (van Driel, 1980). This would lead to a symmetry mismatch between the sixfold axis of the core structure and the 5-fold axis of the outer capsid, arguing against specific binding interactions between the two. These results led Casjens and Hendrix (1988) to suggest that specfic subunit interactions are less important than global properties of the entire scaffold surface, such as charge or hydrophobicity.

On the other hand, a mutation in the T4 coat protein, gp23, that caused the production of giant phage could be supressed by pairs of mutations in the major scaffolding gp22 and the vertex protein gp24 (Doherty, 1982a). This supression was allele specific (Doherty, 1982b), implying that some specific protein-protein interactions between gp23 and gp22/gp24 are involved in assembly of T4.

**Initiation and portal incorporation**

While the theory of quasi-equivalence can explain the structure of icosahedral virus capsids, it cannot explain how non-icosahedral features are inserted. An unsolved question of phage assembly is how the virus ensures the incorporation of a portal complex at one, and only one, of twelve equivalent five-fold vertices. Scaffolding proteins appear to play a critical role in this mechanism, since the aberrant coat protein structures formed in the absence of scaffolding do not contain the portal protein (Earnshaw and King,

1978; Ray and Murialdo, 1975). Assembly of the portal complex is believed to be an early step in phage assembly (Murialdo and Becker, 1978a), suggesting that scaffolding protein may be involved in procapsid initiation.

Early events in phage assembly have been carefully studied in phage lambda (Murialdo and Becker, 1978b; Murialdo, 1979; Kochan and Murialdo, 1983). The initial structure formed in assembly of lambda procapsids is a complex of gpB, the portal protein, with the host encoded chaperone GroE. Although this complex does not contain the lambda scaffolding protein, the presence of functional scaffolding is essential for formation of these initiation complexes. Murialdo (1979) proposed that the scaffolding might function as a "bridge" between the portal and the coat protein.

Consistent with such a function, direct interactions between the phi29 scaffolding and portal protein have been observed *in vitro* by gel shift assays on native gels (Guo et al., 1991). A missense mutation in the P22 scaffolding protein causes assembly of procapsids lacking portals (Bazinet and King, 1988), and may identify a region involved in mediating portal interactions. The scaffolding protein of T4 appears able to interact directly with the portal while the coat protein cannot. When T4 portal and scaffolding proteins are mixed in the absence of functional coat, naked cores are formed that include the portal protein (van Driel and Couture, 1978b). When portal and coat proteins are present but scaffolding is absent, however, polytubes are made that do not incorporate the portal protein (van Driel and Couture, 1978b).

As noted above, the 6-fold symmetry of the T4 scaffolding core does not correspond to the 5-fold axis of the capsid, but it does match the 12-fold symmetry of the portal. Thus the portal may be stably attached to the procapsid by the scaffolding protein but become free to rotate during DNA packaging after the scaffolding has been removed.

**DNA packaging and capsid expansion**

Before the DNA can fill a capsid, up to hundreds of molecules of scaffolding protein must be released. The three-dimensional image reconstruction of the P22 procapsid reveals channels of approximately 25 Å diameter at the six-fold centers (Prasad et al., 1993; Fig. 1.2b), which are presumably the ports for scaffolding exit. The lambda procapsid does not have similar channels, perhaps because the lambda scaffolding is cleaved into small fragments (Ray and Murialdo, 1975) and does not require large holes for exit. It was originally thought that scaffolding release might be energetically coupled to DNA entry (King et al., 1973; King and Casjens, 1974). For phages lambda and T4, however, the substrate for DNA packaging is a procapsid already empty of DNA (Hohn et al., 1975; Hsiao and Black, 1977), indicating that in these phages the two processes are not coupled. Laemmli and Favre (1973) proposed that charged fragments of the T4 scaffolding protein remaining in the head after cleavage play a role in DNA condensation. Since the scaffolding proteins of P22, T7, and phi29 exit intact without leaving any fragments behind (King et al., 1973; Roeder and Sadowski, 1977; Nelson et al., 1976), this cannot be a general mechanism either. There is currently no evidence that scaffolding plays any active role in DNA packaging (Black et al., 1989).

The proteolysis of scaffolding normally preceeds expansion in T4 and lambda (Black et al., 1994; Zachary and Simon, 1977). While P22 procapsids can be expanded *in vitro* by treatment with heat (Galisteo and King, 1993) or SDS (Earnshaw et al., 1976) these treatments also extract the scaffolding protein. In no case have expanded capsids containing scaffolding been observed, implying that either the expansion forces out the scaffolding, or that scaffolding must release from the procapsid before it can expand.

### Scaffoldings as multifunctional proteins

The results discussed above suggest a role for scaffolding proteins in both capsid inititation and incorporation of minor proteins as well as morphogenesis of the capsid. This implies that scaffolding proteins are able to make several distinct types of interactions: with coat proteins, with portal proteins, and with other scaffolding molecules.

These functions may reside on distinct regions of the molecule. A long amber mutant of the T4 major scaffolding assembles naked cores, while the coat protein forms polyheads as though the scaffolding were not present (Traub and Maeder, 1984). The authors proposed that the C-terminus was required to mediate binding of the scaffold to the coat protein, while the sites required for self-association were in a separate domain. A temperature-sensitive mutant of the P22 scaffolding can assemble procapsids at the normal rate, but these procapsids entirely lacked portal protein (Bazinet and King, 1988), demonstrating that the coat and portal interactions are separable functions.

The study of viral scaffolding proteins may therefore help to dissect events of the viral assembly pathway, not only for the phage, but also for the more complex animal viruses.

## SCAFFOLDING PROTEINS OF ANIMAL VIRUSES

### Herpesviruses

The herpesviruses (reviewed in Roizman, 1991) are a class of over 100 animal viruses that cause a variety of infections in both animals and people. These viruses are distinctive in their ability to remain latent within the host for life, and to reactivate to cause lesions near the original site of infection. Herpesviruses consist of icosahedral capsids of approximately 100-110 nm in

diameter, containing 162 capsomeres, surrounded by a tegument and a membranous envelope with virally encoded glycoprotein spikes. Their genomes are linear and double stranded, ranging from 120 to 230 kilobasepairs. The synthesis of DNA and assembly of capsids occur in the nuclei of infected cells, and the capsids are obligatorily enveloped as they exit the nuclear membrane. Mature virions contain from 30 to 35 proteins.

The assembly pathways of herpesviruses have proven to bear strong resemblances to those of the dsDNA phages, in that they too package DNA into preformed capsids that are assembled with the aid of scaffolding proteins. During infection with herpesvirus, cells accumulate three classes of virus particles, referred to as A, B, and C capsids (Gibson and Roizman, 1972). The C capsids contain DNA and mature into infectious virions (Perdue et al., 1976), while A and B capsids lack DNA.

Studies of infected cells in which DNA packaging has been blocked by either temperature-sensitive mutations (Preston et al., 1983; Sherman and Bachenheimer, 1988) or drugs (Lee et al., 1988) result in the accumulation of B capsids. The B capsids are partially penetrated by stain, but include an internal structure that resembles an interior ring as seen in thin sections of infected cells (Lee et al., 1988; Preston et al., 1983). The B capsids made by certain ts mutants are observed to chase to virions after a temperature shift (Preston et al., 1983), confirming them as an on-pathway intermediate. The A capsids are an off-pathway intermediate, equivalent to the "empty heads" sometimes purified from phage infected cells, which result from abortive attempts at DNA packaging (Sherman and Bachenheimer, 1988).

The B capsids of herpes simplex virus 1 (HSV-1) contain all the proteins found in A capsids, as well as two additional proteins, VP22a and VP21 (Gibson and Roizman, 1972). While VP21 is found in C capsids, VP22a

(also referred to as ICP35) is not (Gibson and Roizman, 1972). Similar proteins, often termed assembly proteins, have been found in the B capsid equivalents of several other members of the herpesvirus family. These proteins make up the second largest component by mass of the B capsids, following the major capsid protein. The VP22 of equine herpesvirus (EHV) is present in approximately 600 copies per capsid, or about a 2:3 ratio to the capsid protein (Newcomb et al., 1989), while HSV-1 VP22a is present in nearly twice that amount (Newcomb and Brown, 1989; 1991).

The VP22a has been located in the capsid interior, consistent with a role as an internal scaffold. EHV B capsids have been shown by cryo-EM and image reconstruction to be morphologically identical to A capsids, except for the presence of internal density within the B capsid (Baker et al., 1990). Since EHV B capsids differ in composition from A capsids only in the presence of VP22, this protein probably forms the core structure. Extraction of purified HSV-1 B capsids with 2 M GuHCl removes all the VP22a, and also removes the core structure (Newcomb and Brown, 1991).

The herpesvirus assembly proteins (which I will now refer to as scaffolding proteins) share certain common features (Stinski, 1991): all are about 35-40 kD in size, are phosphorylated, and undergo proteolytic processing. The scaffolding substrate is encoded within the protease coding region with its own start site (Liu and Roizman, 1991, Welch et al., 1991a), so that the scaffolding sequence is identical to the C-terminal portion of the protease. The N-terminal region of the protease contains the proteolytic activity (Liu and Roizman, 1992; Welch et al., 1991b) and displays considerable homology across members of the herpesvirus family. There is also some homology to the bacteriophage T4 protease (Welch et al., 1991b).

During assembly, the protease cleaves itself to liberate the N-terminal proteolytic domain (Deckman et al., 1992). It also cleaves 25 amino acids from its C-terminus, as well as from the identical site on the scaffolding protein (DiIanni et al., 1993). Both the N-terminal and C-terminal domains of the protease remain associated with the viral capsid (Weinheimer et al., 1993).

Expression of HSV-1 capsid proteins in baculovirus systems has permitted the characterization of each protein's role in B capsid assembly (Thomsen et al., 1994; Tatman et al., 1994). In the absence of the VP22a coding region, gene 26.5, the other capsid proteins still assemble some normal B capsids. In the absence of both VP22a and the protease, the remaining proteins form aberrant structures reminiscent of the spirals resulting from amber mutations of phage scaffolding proteins. Similar results were obtained with mutant viruses designed to have null mutations in either the VP22a coding region (Matusick-Kumar et al., 1994) or the entire protease coding region (Desai et al., 1994). These results suggest that the herpes scaffolding is required to guide assembly of closed shells, but that its function can be substituted by the C-terminal region of the protease.

Purified VP22a can form regular structures. These structures are toroidal and resemble the internal rings seen within B capsids (Newcomb and Brown, 1991). While these scaffolding/scaffolding interactions are no doubt important for procapsid assembly, the coat and scaffolding proteins probably copolymerize *in vivo*. Recent experiments have used extracts of cells infected with recombinant baculoviruses coding for HSV-1 capsid proteins to study the HSV assembly pathway *in vitro* (Newcomb et al., 1994). The first structures observed by electron microscopy are double-layered "arcs," similar to those seen in the initial stages of P22 *in vitro* assembly (Prevelige et al., 1993). VP22a is required for localization of the HSV-1 major capsid protein,

VP5, to the cell nucleus (Matusick-Kumar et al., 1994; Nicholson et al., 1994), indicative of early interactions between the two proteins.

Cleavage of the C-terminal 25 amino acids from the herpes scaffolding protein is essential for production of mature virions. The ts mutation which blocks this cleavage (Preston et al, 1983) results in accumulation of B capsids and unpackaged DNA. In cells expressing only altered forms of both scaffolding and protease lacking the C-terminal region, the resultant structures were aberrant capsids similar to those produced in the complete absence of the scaffolding sequence (Thomsen et al., 1995; Matusick-Kumar et al., 1995). This C-terminal region must be essential for assembly. This region does not seem to be required for scaffolding/scaffolding interactions, since cleaved protein can form cores *in vitro* (Newcomb and Brown, 1991) and *in vivo* (Kennard et al., 1995). The C-terminus is probably required to mediate binding to the coat protein, since antibodies to VP5 were unable to coprecipitate the truncated VP22a (Thomsen et al., 1995). Cleavage of this end would allow the rest of the molecule to exit, permitting the DNA to enter.

It is not known how the DNA enters the capsid; while there may be a portal protein it has not been identified, and the methods used to reconstruct capsid images would not reveal the presence of a structure present at only one vertex. Given the already striking similarities between the herpes and phage assembly pathways, it appears likely that this feature is conserved as well.

**Adenoviruses**

Members of the adenovirus class (reviewed in Horwitz, 1991) are icosahedral, non-enveloped viruses of 60-80 nm in diameter that contain a dsDNA genome of approximate molecular weight $24x10^6$ kD. The adenovirus capsid is composed of 252 capsomeres arranged in a T=25 lattice, with 240

hexons and 12 pentons at the vertices. A structure known as the fiber projects from each vertex. Mature capsids contain from 11 to 15 distinct polypeptides, of which the major component is the hexon protein. Each hexameric capsomere is composed of a trimer of the 120 kD hexon protein.

While the adenovirus assembly pathway is complex, several well-defined intermediates have been identified, and their order in the assembly pathway determined by pulse-chase experiments and the characterization of mutants blocked at the various steps (Edvardsson et al., 1976; D'Halluin et al., 1978a; b). The first is the light intermediate. This particle contains hexon, proteins pVI, pVIII, IIIa, and phophorylated proteins of 50 and 39 kD, along with a 7-11S DNA fragment. The 39 and 50 kD proteins are lost concurrently with DNA packaging to form the heavy intermediate particle, which contains the full 34 S DNA genome. The next intermediate, the young virion, has incorporated the core proteins V, pVII and IX. During the transition from the young virion to mature virus, the precursor proteins are cleaved, their fragments released or degraded, and the particle configuration tightens, becoming more resistant to nucleases.

When observed in the electron microscope, the light intermediate particles resemble the procapsids of bacteriophages, in that they are rounder and rougher edged than later intermediates or mature viruses, lacking distinct icosahedral edges (D'Halluin et al., 1978b). These particles are partially penetrated by stain, but have an internal structure resembling a second internal ring, similar to that seen in the interior of herpesvirus B capsids, or in phage capsids observed in thin sections. The heavy intermediate, in contrast, has a defined icosahedral shape, although the vertices are less distinct than in the mature virus, and a dense interior, presumably due to the DNA. Young virions are identical in morphology to mature viruses.

The light intermediate particles resemble both the bacteriophage procapsids and the herpesvirus B capsids in having a distinctive morphology from mature virions, lacking DNA, and possessing an internal core structure. These intermediates also contain significant amounts of proteins not found in later intermediates, the 39 and 50 kD proteins, which were initially proposed to be scaffolding proteins (D'Halluin et. al., 1978b; Edvardsson et al., 1976), since their exit from the capsid coincides with DNA packaging.

The identities of the 39 and 50 kD phosphorylated proteins have long been obscure. The 50kD protein was proposed to be the virion protein VIa2 (Persson et al., 1979), but later experiments with monoclonal antibodies to this protein demonstrated that, unlike 50kD, IVa2 was found in young virions and mature viruses as well as intermediate particles (Winter and D'Halluin, 1991).

The L1 region of the adenovirus genome, which codes for the virion protein IIIa, also encodes a 48kD protein that is alternatively phosphorylated to give 52 and 55 kD products (Lucher et al., 1986). Monoclonal antibodies to this protein found it present in 50-100 copies in intermediate particles, but in only 1-2 copies in young virions and not at all in mature virions (Hasson et al., 1992). The antibodies also recognized a 40 kD protein that appeared to be generated as a proteolytic fragment of the 52/55 kD protein. If the 40kD fragment is included, light intermediate capsids contain over 100 copies of the 55/52 kD protein and its products.

It appears likely that the 55/52 kD protein is the 50 kD protein (observed to be a doublet by Morin and Boulanger, 1984), while its 40 kD fragment is the 39kD protein. Consistent with 55/52 kD being a scaffolding protein, a temperature-sensitive mutant generated within its coding region resulted in the accumulation of particles resembling the light intermediate

(Hasson et al., 1989). All the monoclonal antibodies to 55/52 kD are unable to precipitate the protein unless the capsids have been previously disrupted (Hasson et al., 1992). As the antibodies all recognize the N terminus of the protein, at least this portion of the molecule, or perhaps all of it, must reside in the interior of the capsid, consistent with a role as a scaffolding core.

The adenoviruses thus appear to resemble the herpesviruses in possessing a scaffolding protein that is phosphorylated and proteolytically processed. In contrast to the herpesviruses, the adenovirus protease does not include the scaffolding protein sequence, but is encoded by a separate gene (Anderson, 1990). The role of the proteolytic cleavages of the scaffolding protein in the assembly reaction is unknown, as no mutants affecting this step have been isolated in adenoviruses. The portal protein, if there is one, and its interactions with the scaffolding, remain to be identified.

While many questions remain to be answered for the animal virus systems, new methodologies such as site-directed mutagenesis and image reconstruction techniques have made these systems increasingly tractable. Recent progress on the herpesvirus assembly pathway, discussed above, has been striking. The emerging similarities between the phage and animal viruses are sufficient to insure that phage remain a useful model system for animal virus assembly as well as for more basic questions of protein-protein interactions and morphogenesis.

## SCAFFOLDING PROTEINS OF SINGLE-STRANDED DNA PHAGE

Bacteriophage ΦX174 (reviewed in Hayashi et al., 1988) is an icosahedral virus of the class Microviridae. Its complete genetic sequence (Sanger et al., 1977) and three-dimensional structure are known (McKenna et al., 1992; 1994), making this phage a particularly amenable system for study. This phage

differs from the viruses discussed above, in having a much smaller T=1 capsid and containing single-stranded DNA. Nonetheless, it too packages its DNA into a preformed procapsid shell (Fujisawa and Hayashi, 1977c; Mukai et al., 1979), and its assembly process requires proteins not found in the mature virion (Fujisawa and Hayashi, 1977a; Mukai et al., 1979).

In contrast to the assembly pathways of dsDNA phage or herpesvirus, which involve the addition of monomers onto a growing shell, the first step in the assembly of ΦX174 is the formation of pentamers of both the coat protein, gpF and the spike protein, gpG. These pentamers join to form a 12S complex, twelve of which form a procapsid (Hayashi et al., 1988). In addition to 60 molecules each of gpF and gpG, the procapsid also contains 60 molecules of gpB and 240 molecules of gpD, neither of which is found in the mature virion (Mukai et al., 1979).

GpB is required for the formation of 12S particles (Siden and Hayashi, 1974; Ekechukwu and Fane, 1995). Although in the absence of functional gpB the 12S particle does not assemble, gpB is not found associated with this structure, but only in the assembled procapsid (Siden and Hayashi, 1974). The copy number of gpB suggests a 1 to 1 interaction with the coat protein, unlike the situation in the dsDNA viruses. GpB is lost from the procapsid at some point during DNA packaging (Siden and Hayashi, 1974; Mukai et al, 1979). Surprisingly, phage with mutations in gpB grown under permissive conditions are less stable to heating (Sinsheimer, 1968; Siegel and Hayashi, 1969). Since gpB is not part of the mature phage, the bonds holding together either the 12S particle or the assembled capsid may be improperly formed in the presence of the mutant protein.

GpD is not required for assembly of 12S particles (Fujisawa and Hayashi, 1977a), but associates with them during the formation of the

procapsid (Fujisawa and Hayashi, 1977a,c). GpD is a tetramer in solution (Farber, 1976), suggesting that tetramers of gpD may bind to each coat molecule. In distinct contrast to the dsDNA scaffolding proteins, gpD is located on the outside of the procapsid (Fujisawa and Hayashi, 1977a; b), forming an external rather than an internal scaffold. This scaffold remains associated with a 132S particle after DNA packaging. The gpD is removed to yield the 114S mature virus in a process that involves conformational changes in the viral surface (Fujisawa and Hayashi, 1977b).

Why should such a simple virus require not one, but two scaffolding proteins for its assembly? Their role may be not so much capsid assembly as capsid stabilization. Amber or cold-sensitive mutants in gpD result in accumulation of 12S particles (Fujisawa and Hayashi, 1977a; Fane et al., 1993). However, the cold-sensitive gpD strain could produce procapsids when DNA packaging was blocked, suggesting that the protein may not be required for procapsid formation, but to stabilize the procapsid and prevent it from exploding under the stresses of DNA packaging (Ekechukwu and Fane, 1994).

Second site suppressors of cold-sensitive mutations in both gpB and gpD have been mapped to the coat protein gpF (Fane and Hayashi, 1991; Fane et al, 1993). The suppressor mutations reside in regions of the coat protein located near the threefold axes of symmetry (McKenna et al., 1994). Assuming that the procapsid assembles from 12 pentamers of coat and spike proteins, the threefold axes would represent junction sites between pentamers. Thus the suppressor mutations might add extra stability to the capsid to compensate for absent or defective scaffolding proteins.

Given the differences in assembly pathways, it is unlikely that the ΦX174 scaffolding proteins make precisely analogous interactions to those of the dsDNA viruses. It is therefore striking that the use of scaffoldings is

conserved, and suggests that the presence of scaffolding proteins might be a necessary feature of all pathways that involve DNA packaging into a procapsid intermediate.

# CHAPTER 2

# BINDING OF SCAFFOLDING SUBUNITS WITHIN THE P22 PROCAPSID LATTICE

## INTRODUCTION

Substantial progress has been made in determining the structures of viruses by crystallography or cryo-electron microscopy and image reconstruction, but the detailed pathways by which these structures are formed from coat protein subunits remain unclear. For dsDNA viruses such as bacteriophage (Casjens and Hendrix, 1988), herpesviruses (Lee et al., 1988; Sherman and Bachenheimer, 1988) and adenoviruses (D'Halluin et al., 1978; Edvardsson et al., 1976), the initial product of subunit polymerization is not the mature virion but a precursor capsid, into which DNA is packaged. These procapsids contain proteins not found in the mature virion but required for correct assembly of coat subunits into closed precursor shells, termed scaffolding proteins (King and Casjens, 1974; Casjens and Hendrix, 1988).

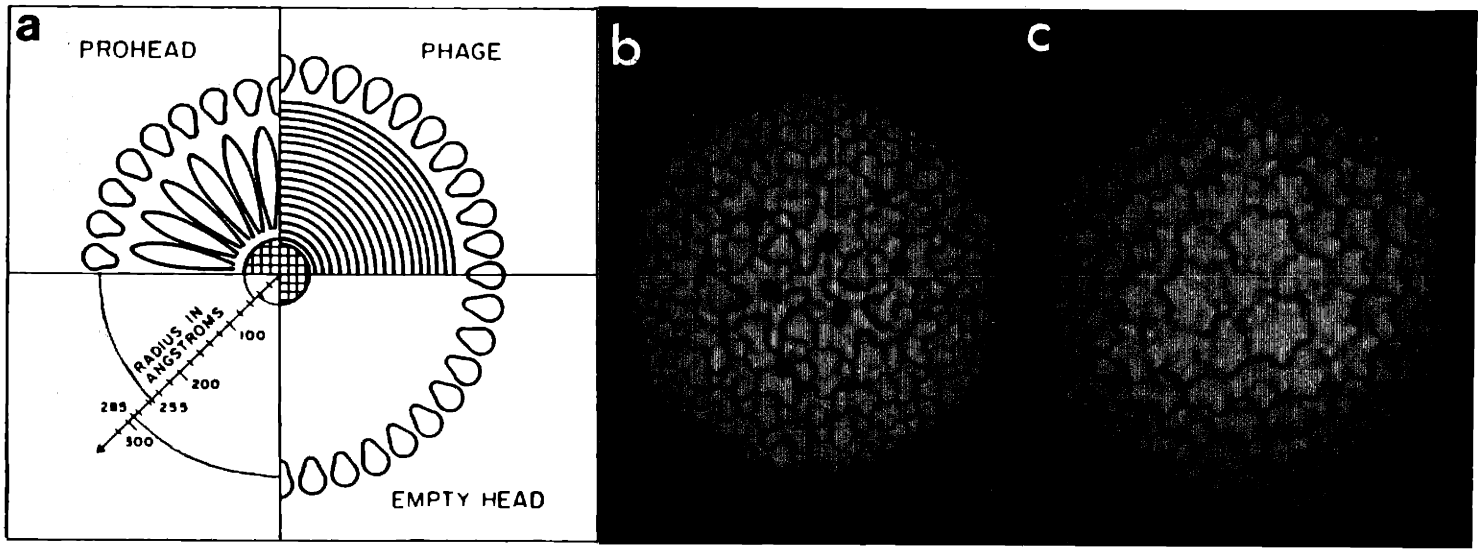
For P22, a bacteriophage of *Salmonella typhimurium*, intracellular assembly of procapsids involves 420 molecules of coat protein and approximately 300 scaffolding subunits which copolymerize along with 12 molecules of the portal protein and 10-20 molecules each of three pilot proteins required for DNA injection (King et al., 1973). The structure of procapsids and mature phage have been compared by low angle x-ray scattering (Fig. 2.1a, Earnshaw et al., 1976) and cryo-electron microscopy (Fig. 2.1b and c, Prasad et al., 1993). The procapsid structure contains no DNA, but

includes an inner shell of scaffolding subunits. The coat subunits are arranged in a T=7 lattice with prominent channels at the centers of skewed sixfold clusters. The arrangement of the scaffolding within the procapsid has not been determined for P22 or for any other virus.

During the process of phage maturation, all 300 scaffolding molecules exit the procapsid intact, presumably through the channels in the coat lattice, to participate in further rounds of procapsid assembly (King and Casjens, 1974; Casjens and King, 1974). The DNA is driven into the shell by ATP hydrolysis and the shell lattice undergoes conformational changes resulting in expansion, angularization, and closure of the channels (Prasad et al, 1993). Within cells, scaffolding release is closely coupled to DNA packaging (Earnshaw and Casjens, 1980). After assembly the procapsid is a stable structure that does not spontaneously lose its scaffolding during months of storage; the changes in the coat:scaffolding interactions that permit rapid release of the 300 scaffolding molecules remain unknown.

I have used low concentrations of the ionic denaturant guanidine hydrochloride (GuHCl) to reproduce the release of scaffolding from procapsids *in vitro* in the absence of DNA (Fuller and King, 1981). This release was reversible, permitting study of the binding of scaffolding within the assembled procapsid lattice and other coat protein structures.

FIG. 2.1 (Same as Fig. 1.2) Structure of the P22 procapsid. a) structure of the procapsid (prohead), mature phage, and empty mature phage (empty head) as determined by x-ray scattering (Earnshaw et al., 1976). Cryo-EM image reconstruction of b) extracted procapsid shell, and c) phage (Prasad et al., 1993).


a
PROHEAD
PHAGE
RADIUS IN ANGSTROMS
100
200
285
235
300
EMPTY HEAD
b
c

## MATERIALS AND METHODS

### Preparation of coat structures

Procapsids, empty procapsid shells and scaffolding monomers were prepared as described (Prevelige et al., 1988). Briefly, *Salmonella* cells were infected with P22 phage carrying an amber mutation in gene 2, whose product is required for DNA packaging (Poteete et al., 1979). After 3 1/2 hours, the cells were pelleted by centrifugation, lysed by repeated freeze/thaw cycles, and treated with DNase and RNase. Cell debris was removed by low speed centrifugation, and the procapsids were collected by high speed centrifugation. A portion of the procapsids were extracted with 0.5 M GuHCl, and purifed by size-exclusion chromatography in 0.5 M GuHCl to yield empty procapsid shells (Fuller and King, 1981). Extracted scaffolding protein was concentrated by ammonium sulfate precipitation and purified from minor proteins by chromatography on a DEAE 52 column.

Mature phage heads empty of DNA were prepared using a phage bearing an amber mutation in gene *10*, required for stabilization of packaged DNA (Strauss and King, 1984). Cells were infected and structures collected as described above. Empty heads were separated from procapsids and full heads by centrifugation through 15-30% sucrose gradients for 4.5 hours at 24,000 rev/min in a SW27.1 rotor. The bands containing empty heads were removed with a syringe and dialysed to remove the sucrose.

Aberrant coat structures were prepared from cells infected with the mutant phage *8amH202*, which does not produce the scaffolding protein. This mutation results in the production of large spiral structures, shells the size of procapsids but empty of scaffolding (also referred to as $8^-$ shells), and small filled shells (Earnshaw and King, 1978). These structures were collected as

described above, then chromatographed on a Bio-Gel A 50-m column. Fractions containing predominantly either spirals or empty shells, as determined by electron microscopy, were pooled and concentrated by high-speed centrifugation. Spirals were also generated at much lower levels in the *2am* infection. These spirals were separated from procapsids by centrifugation through 5-20% sucrose gradients for 25 minutes at 35,000 rev/min in a SW50.1 rotor. Fractions from the bottom third of the gradient were pooled, dialysed to remove sucrose, and combined with the spirals obtained in the *8am* infection. The resultant preparations were approximately 80% pure, as assayed by electron microscopy, with some contamination of spirals by procapsids and shells, and of empty shells by the small filled shells.

Concentrations of all stock solutions were determined by absorbance at 280 nm, using extinction coefficients of 1 for coat protein and 0.45 for scaffolding in 1 mg/ml solutions (Fuller and King, 1981). Coat structures were dissociated with 3 M GuHCl to prevent contributions of scattering to the signal. Coat structures were stored at 4°C at concentrations ranging from 2 mg/ml for spirals to 20 mg/ml for procapsids. Scaffolding protein was stored as frozen aliquots at 4 mg/ml.

**Sucrose gradient sedimentation and SDS-PAGE**

Procapsids to be extracted were incubated overnight at 1.5 mg/ml in varying concentrations of buffered GuHCl. Samples of 200 µl were then centrifuged through 5 ml 5-20% (w/w) sucrose gradients for 35 minutes at 35,000 rev/min in a SW50.1 rotor at 20°C. The sucrose solutions were made up in the same GuHCl concentrations as the samples to prevent any possible reentry of scaffolding during centrifugation. Samples treated with salts were similarly centrifuged through gradients containing that salt. Gradients were

fractionated into 18 fractions through a pinhole at the bottom of the tube. For reentry experiments, various amounts of scaffolding were mixed with coat structures present at 1.0 mg/ml. Samples were centrifuged as above, except for spirals, which were centrifuged for only 20 minutes. Either 18 or 30 fractions were collected. In all cases, fractions were concentrated by TCA precipitation and resuspended in sample buffer in 0.67 M Tris. Samples were analysed by SDS-PAGE in a slab gel apparatus and stained by Coomassie blue. Gels were quantified on an LKB laser densitometer.

**Kinetic measurements**

Kinetics of scaffolding reentry into empty coat shells was monitored by increase in light scattering using a Hitachi F4000 fluorescence spectrophotometer interfaced to a personal computer. The excitation and emission wavelengths were both set to 500 nm, excitation and emission slit widths to 5 nm, and the PMT voltage to 400. Empty shells were added to a thermostated cuvette at 25°C to give a coat protein concentration of 50 μg/ml. A constant volume of scaffolding protein at a concentration calculated to give a final scaffolding:coat protein mass ratio from 1:1 to 1:4 was added. The dead time between opening the cuvette holder and the start of data collection was approximately 10 seconds. Relaxation times were determined with the Kaleidagraph software (Abelbeck) using a first order rate equation with either one or two exponentials.

**Electron microscopy**

Samples were deposited on carbon coated copper grids, negatively stained by 2% uranyl acetate, and air dried. The grids were examined in a JEOL 100B electron microscope at 80 kV.

## RESULTS

### Extraction of scaffolding from procapsids by GuHCl

Scaffolding subunits can be efficiently extracted from P22 procapsids by 0.5 M GuHCl (Fuller and King, 1981; Prevelige et al., 1988). In order to characterize the range of GuHCl capable of extraction, procapsids were incubated overnight in GuHCl concentrations varying from 0.1 to 1.0 M. Samples were centrifuged on sucrose gradients made up to the same GuHCl concentration as the sample, to prevent alteration of the distribution of scaffolding protein during centrifugation. Scaffolding protein sedimenting with the procapsid shells, about a third of the distance from the bottom of the gradient, and subunits from the top of the gradient were quantified and compared for each sample, as shown in Fig. 2.2.

Untreated procapsids did not spontaneously lose their scaffolding, and all scaffolding protein was recovered migrating with the capsid peak. In contrast, after incubation in 0.5 M GuHCl, 95% of the scaffolding protein was found at the top of the gradient. Even as little as 0.2 M GuHCl resulted in 17% of the scaffolding being extracted. To test whether this effect was due solely to the high ionic strength of the GuHCl solutions, procapsids were incubated in solutions of various salts, including NaCl, $NH_4Cl$, LiCl, KCl, $MgCl_2$, $(NH_4)_2SO_4$, $Na_2SO_4$ , $MgSO_4$, and $Mg(CH_3CO_2)_2$ at similar and higher ionic strengths. While some of these salts did extract scaffolding, this process was much less effective than with the GuHCl; $MgCl_2$, the most effective of the salts, extracted only 30% of the scaffolding when present at 0.5 M.

No detectable change in the outer shell of coat protein was observed until GuHCl concentrations above 1 M, at which point the shells begin to dissociate into monomers (Teschke and King, 1993).

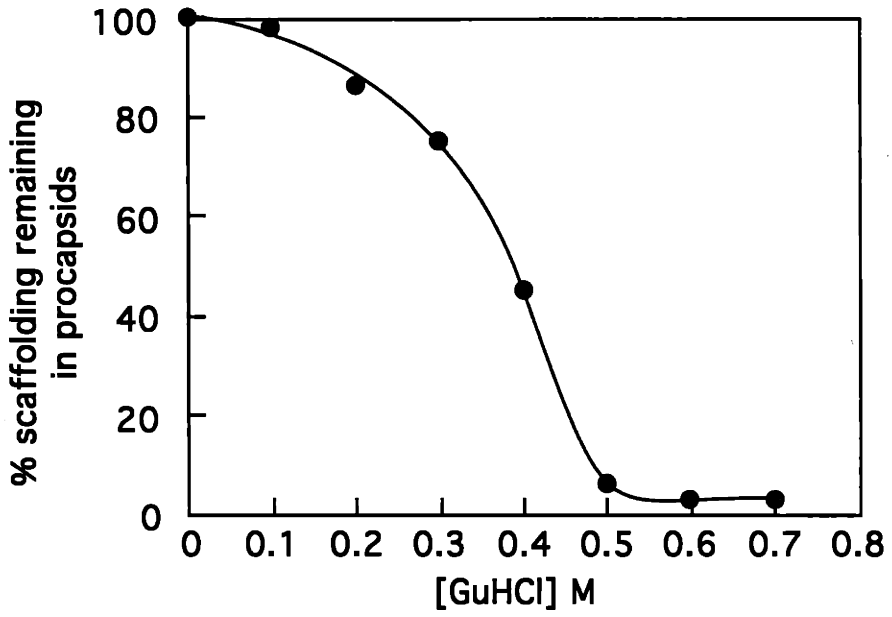



FIG. 2.2 Extraction of scaffolding from procapsids by GuHCl. Procapsids at 1.5 mg/ml were incubated with varied concentrations of GuHCl for 18 hours and the capsids separated from scaffolding monomers by centrifugation through 5-20% sucrose gradients as described in Materials and Methods. Fractions from the gradient were analysed by SDS-PAGE to locate the procapsid proteins. The amount of scaffolding within the capsid and that sedimenting as monomers were quantified by densitometry of the stained gels. Filled circles represent the percentage of total scaffolding protein on each gel that sedimented with the capsid peak.

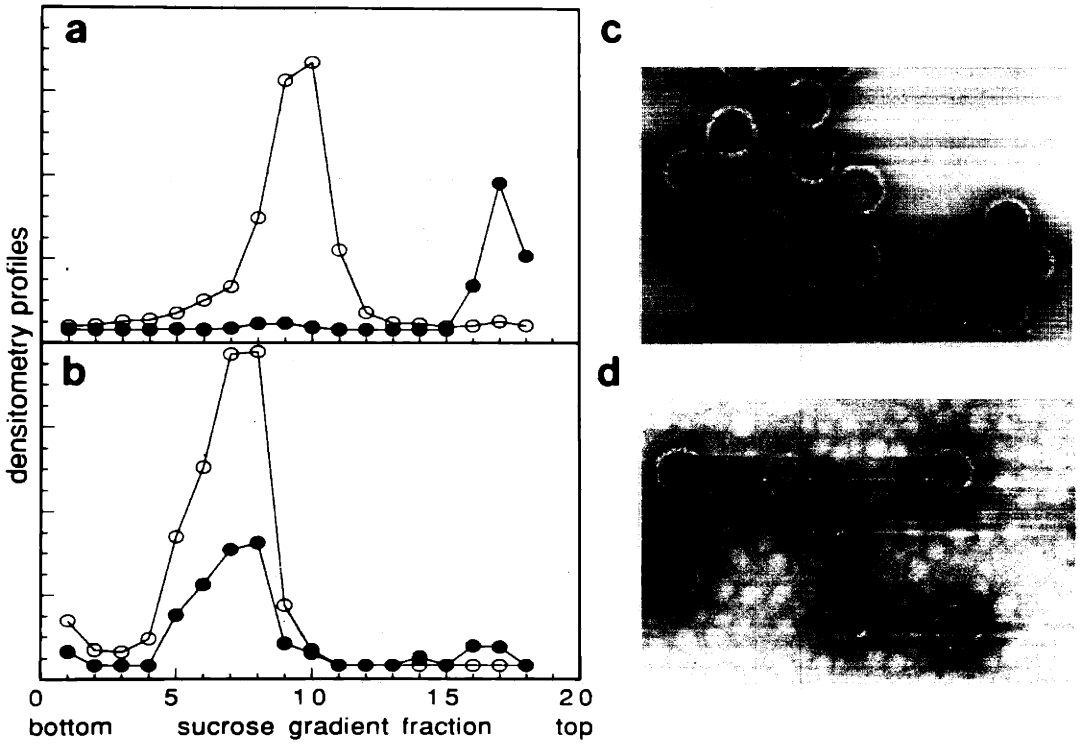
**Reentry of scaffolding upon dialyis of GuHCl**

Since the extracted procapsid shells remained intact and unexpanded, I was curious as to whether scaffolding protein could reenter the procapsid shells in the absence of guanidine. Procapsids in 0.5 M GuHCl, from which the scaffolding protein had been removed (Fig. 2.3a) were dialysed overnight against buffer B. After centrifugation through sucrose gradients, 90% of the scaffolding protein sedimented with the coat shells, the rest remaining at the top of the gradient (Fig. 2.3b). The material in the particle peak had an increased S-value compared to the empty shells, and a scaffolding:coat protein ratio similar to that of procapsids. Electron micrographs showed that after dialysis most of the coat shells had regained the filled centers characteristic of procapsids (Figs. 2.3c and 2.3d). No detectable aggregates of P22 scaffolding were produced, in contrast to what is observed with the HSV protein VP22a, which forms toroid structures upon dialysis from GuHCl (Newcomb and Brown, 1991). Together with the sucrose gradient results these data suggest that all the scaffolding molecules either reenter the coat shells or remain as soluble monomers. The fact that P22 scaffolding, in the absence of free coat protein, does not form any large aggregates that might compete with reentry (Prevelige et al., 1988) may explain why the reentry reaction is much more efficient than for HSV VP22a.

**Reconstitution from purified components**

In order to study the reentry reaction more quantitatively in the absence of GuHCl, empty procapsid shells and scaffolding monomers were separately purified as described under Materials and Methods. Empty procapsid shells were incubated overnight with scaffolding monomers in ratios by mass of coat:scaffolding ranging from 4:1 to 1:1 (as compared to the *in*

FIG. 2.3 Extraction of scaffolding from procapsids by GuHCl and reentry upon dialyis. Procapsids at 1.5 mg/ml were extracted by 0.5 M GuHCl as described. Half of this sample was dialysed overnight to remove GuHCl. Both samples were then centrifuged through 5-20% sucrose gradients, and the resulting fractions analysed by SDS-PAGE. 2 a and b, densitometry profiles from stained gels of sucrose gradient fractions from (a) procapsids extracted with 0.5 M GuHCl, (b) extracted procapsids after dialysis. Filled circles = scaffolding protein, open circles = coat protein. 2 c and d, electron micrographs of (c) extracted procapsids, (d) extracted procapsids after dialysis.


a
b
c
d
densitometry profiles
0
5
10
15
20
bottom
sucrose gradient fraction
top

*vivo* ratio of approximately 2:1). The products were centrifuged through sucrose gradients as above. As shown in Fig. 2.4, the average S-value of the capsids increased linearly with amount of scaffolding protein available up to amounts sufficient to fill the shells; the presence of additional scaffolding did not result in entry of extra scaffolding subunits into the shells. The reentry reaction was somewhat less efficient than in the dialysis experiments, with scaffolding protein in approximately 50% excess of the *in vivo* ratio being required to completely fill the shells.

The products of this reentry reaction were analysed at higher resolution by collecting 30, rather than 18, fractions from each gradient. As shown in Fig. 2.5b, the presence of limiting scaffolding protein resulted in a single peak of shells which migrated more slowly than procapsids. These structures contained less than the amount of scaffolding seen in procapsids produced *in vivo*. The availability of excess scaffolding, as seen in Fig. 2.5d, yielded a single peak with the S-value and protein ratio of procapsids. An intermediate amount of scaffolding (Fig. 2.5c) reproducibly yielded two distinct peaks, one with the S-value and protein ratio of procapsids, and one that migrated more slowly than procapsids, and contained approximately half the amount of scaffolding protein present *in vivo*.

In the assembly of procapsids *in vitro*, as the scaffolding concentration was lowered, the distribution of capsid proteins across the sucrose gradient developed a bimodality similar to that observed here. (Prevelige et al., 1988). These data suggest that there may be two classes of binding sites for scaffolding molecules within the procapsid shell: one set that is filled first and accommodates about half the total number of scaffolding molecules, and a second set that either is not available or not utilized until the first set is filled.

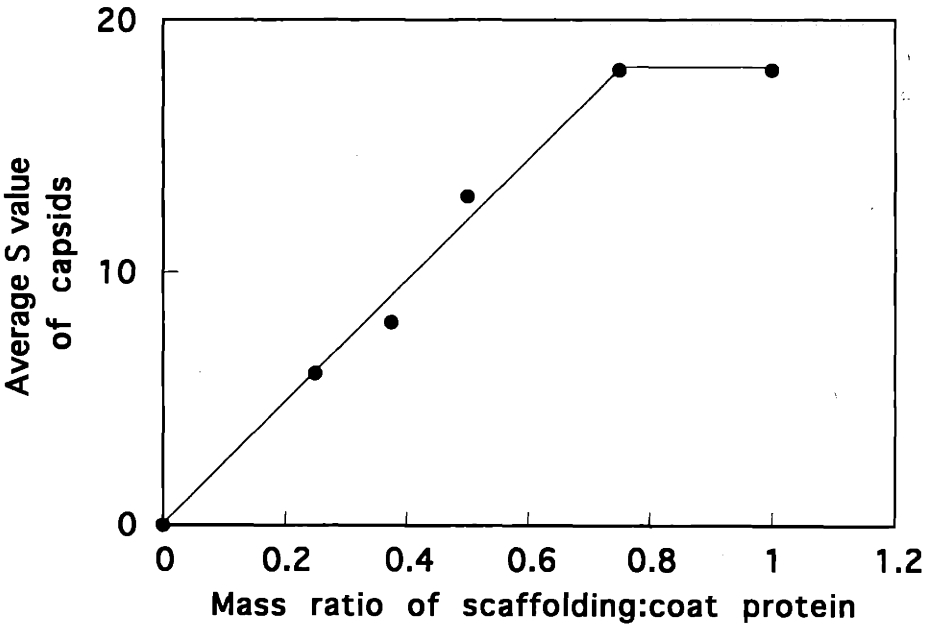
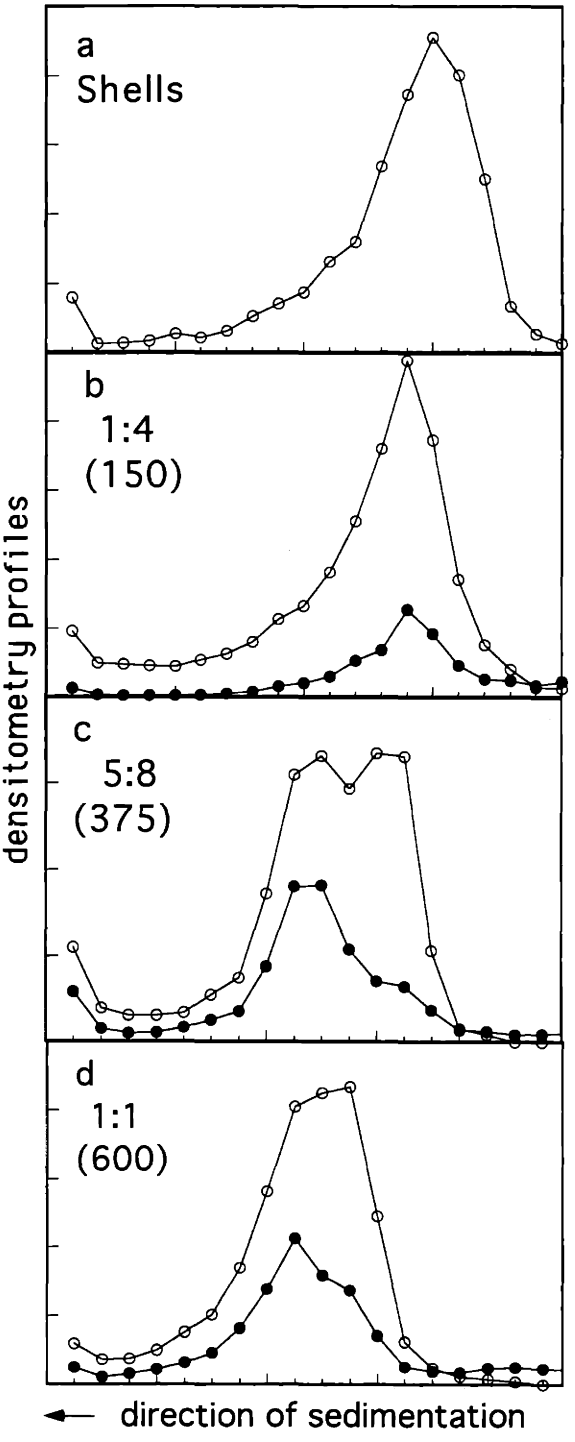



FIG. 2.4 Reentry of scaffolding into procapsids. Purified shells of coat protein were mixed with scaffolding monomers in the indicated proportions and incubated overnight at room temperature. The concentration of coat protein in all samples was 1 mg/ml. Capsids were separated from scaffolding monomers by centrifugation through 5-20% sucrose gradients. Gradients were fractionated and the capsids analyzed by SDS-PAGE. The average S-value of the capsids in each sample was calculated from densitometry of stained gels of the gradient fractions.

FIG. 2.5 Binding of scaffolding to empty procapsids at varied ratios of scaffolding protein to coat protein. Scaffolding monomers were mixed with purified empty procapsids at the indicated mass ratios of scaffolding:coat. Numbers in parentheses indicate the estimated number of scaffolding monomers present per empty capsid in each reaction. The concentration of coat protein in all samples was 1 mg/ml. 200 µl of each mixture was centrifuged through 5-20% sucrose gradients. 30 fractions were collected from each gradient, of which only the bottom 20 are shown here. The fractions were analysed by SDS-PAGE and quantified by densitometry of the stained gels. Filled circles = scaffolding protein, open circles = coat protein. 4a) purified empty capsids, b) empty procapsids plus scaffolding protein for a 1:4 mass ratio of scaffolding:coat, c) 5:8, d) 1:1.


a
Shells
b
1:4
(150)
c
5:8
(375)
d
1:1
(600)
densitometry profiles
direction of sedimentation

**Kinetics of reentry**

To observe the kinetics of refilling, equal volumes of scaffolding monomers at varying concentrations were added to empty procapsid shells under the same conditions as described above. Since filled procapsids scatter light more strongly than empty shells, the kinetics of refilling of procapsids with scaffolding can be monitored by observing the change in light scattering over time. The experiment was performed at least three times at each input ratio, with representative data shown in Fig. 2.6. When scaffolding protein was limiting (curve c), the refilling kinetics were well fit by a single exponential curve with a relaxation time of 61±7 sec. At higher input levels of scaffolding, the kinetics could only be fit by adding a second exponential. The kinetics were similar for both input ratios examined, with relaxation times for the fast phase of 50±5 and 49±6 sec, at input ratios of 1:1 and 5:8 respectively, and relaxation times of 2781±415 and 2616±835 sec for the slow phase. This slow phase is not due to aggregation of the scaffolding protein, since the scaffolding by itself, in the highest concentration used, showed no increase in light scattering over time (curve d). The extraction of scaffolding from procapsids by bis-ANS has also been shown to have biphasic kinetics, with comparable relaxation times of ~30 and ~2500 sec (Teschke et al., 1993).

Given the correlation between the appearance of the second phase and fully filled shells, it is tempting to conclude that the two phases correspond to the production of the two classes of partly and fully filled procapsids observed in the preceeding experiment. Alternatively, the second slow phase could reflect slow conformational changes occuring after large amounts of scaffolding have entered the procapsid.

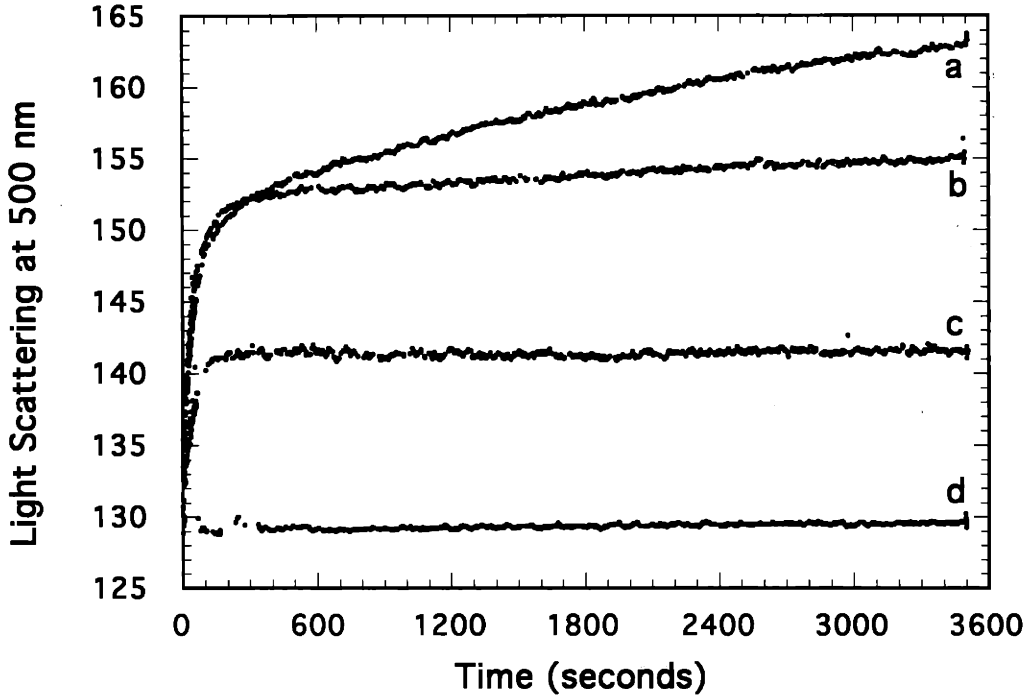



FIG. 2.6 Kinetics of scaffolding reentry into extracted procapsids. Purified scaffolding monomers were mixed with empty procapsids at the indicated ratios by mass of scaffolding:coat protein. The coat protein concentration was constant for all samples at 50 μg/ml. Increase in light scattering with time was monitored at 500 nm. a) empty procapsids plus scaffolding protein for a 1:1 mass ratio of scaffolding:coat, b) 1:2, c) 1:4, d) same amount of scaffolding as in (a), added to buffer. Curve (d) has been moved upwards for easier comparision with the other curves.

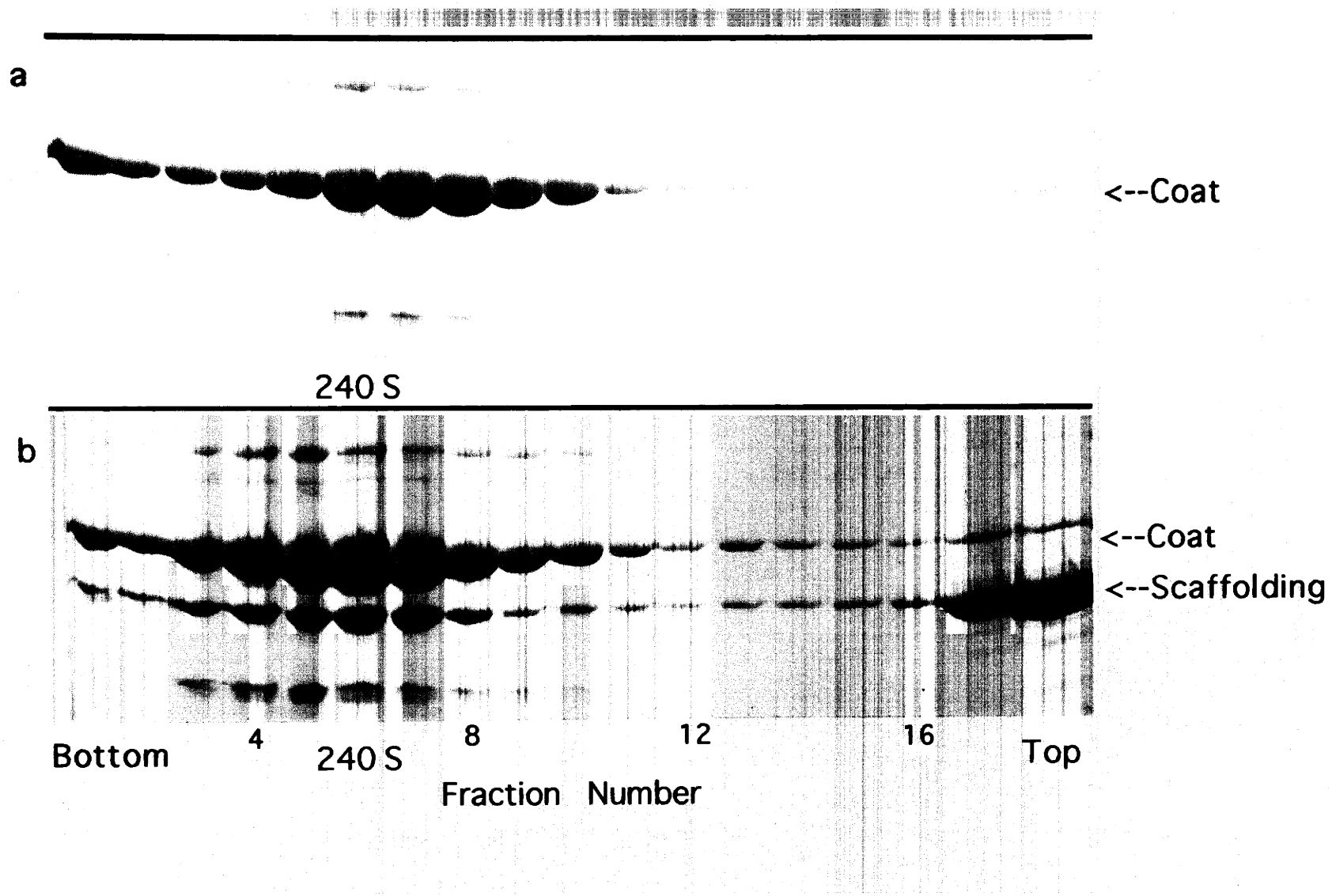
**Binding of scaffolding to coat structures formed in its absence**

The scaffolding subunits bind to specific sites within the coat shell lattice. Can scaffolding recognize and bind coat protein polymerized into aberrant lattices? Such polymers are formed during infections with phage carrying amber mutations in the scaffolding gene. These structures include shells with the dimensions of procapsids but lacking scaffolding protein which I will refer to as *8*$^-$ shells, small shells which appear filled, and large spiral structures (Earnshaw and King, 1978). In order to determine whether scaffolding protein could recognize and bind to coat protein organized in these alternative structures, spirals and *8*$^-$ shells were purified from an *8* amber infection as described. These structures were each mixed with scaffolding protein under conditions which led to complete refilling of empty procapsid shells, and analysed as above.

The *8*$^-$ shells of the size of procapsids, after incubation with scaffolding protein, showed a shift in S-value from that typical of extracted procapsids to that of filled procapsids. (Figs. 2.7a and 2.7b). These filled shells contained scaffolding protein in the ratio found in procapsids. As observed by electron microscopy, the empty *8*$^-$ shells had acquired filled cores similar to those of procapsids. (Figs. 2.8A and 2.8B).

The spirals, after mixing with scaffolding and analysis on sucrose gradients, appeared to bind little scaffolding protein; of the amount available, less than 5% was associated with the major coat peak, the rest sedimenting as monomers. (Figs. 2.7c and 2.7d). Electron microscopy showed that incubation with scaffolding had not altered the appearance of most of the spirals, although a few did display internal density at their centers, where the spiral curvature is closest to that of procapsids. (Fig. 2.8D).

FIG. 2.7 Binding of scaffolding protein to aberrant coat structures. Samples were centrifuged through 5-20% sucrose gradients for 35 min (a,b) or 20 min (c,d) at 35,000 rev/min. The gradients were fractionated and the fractions analysed by SDS-PAGE. The concentration of coat protein and, when present, scaffolding protein, was 1 mg/ml for all samples. The position of normal procapsids, at 240 S, is indicated on the gels, as is the postion of sprials within gradients c and d. 6a) $8^-$ shells, b) $8^-$ shells after incubation with scaffolding protein, c) spirals, d) spirals after incubation with scaffolding protein.


a
<--Coat
240 S
b
<--Coat
<--Scaffolding
Bottom
4
240 S
8
12
16
Top
Fraction Number

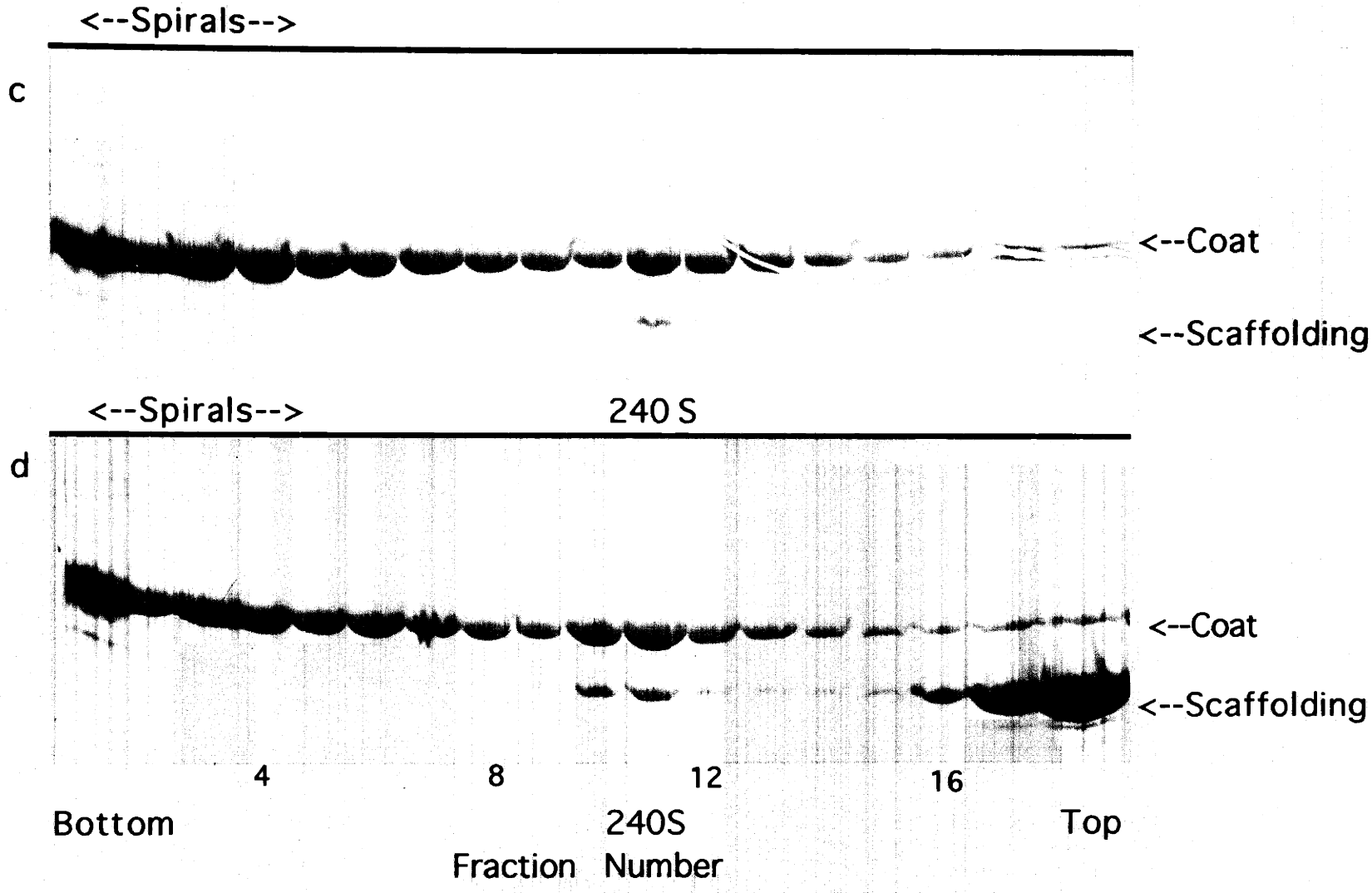
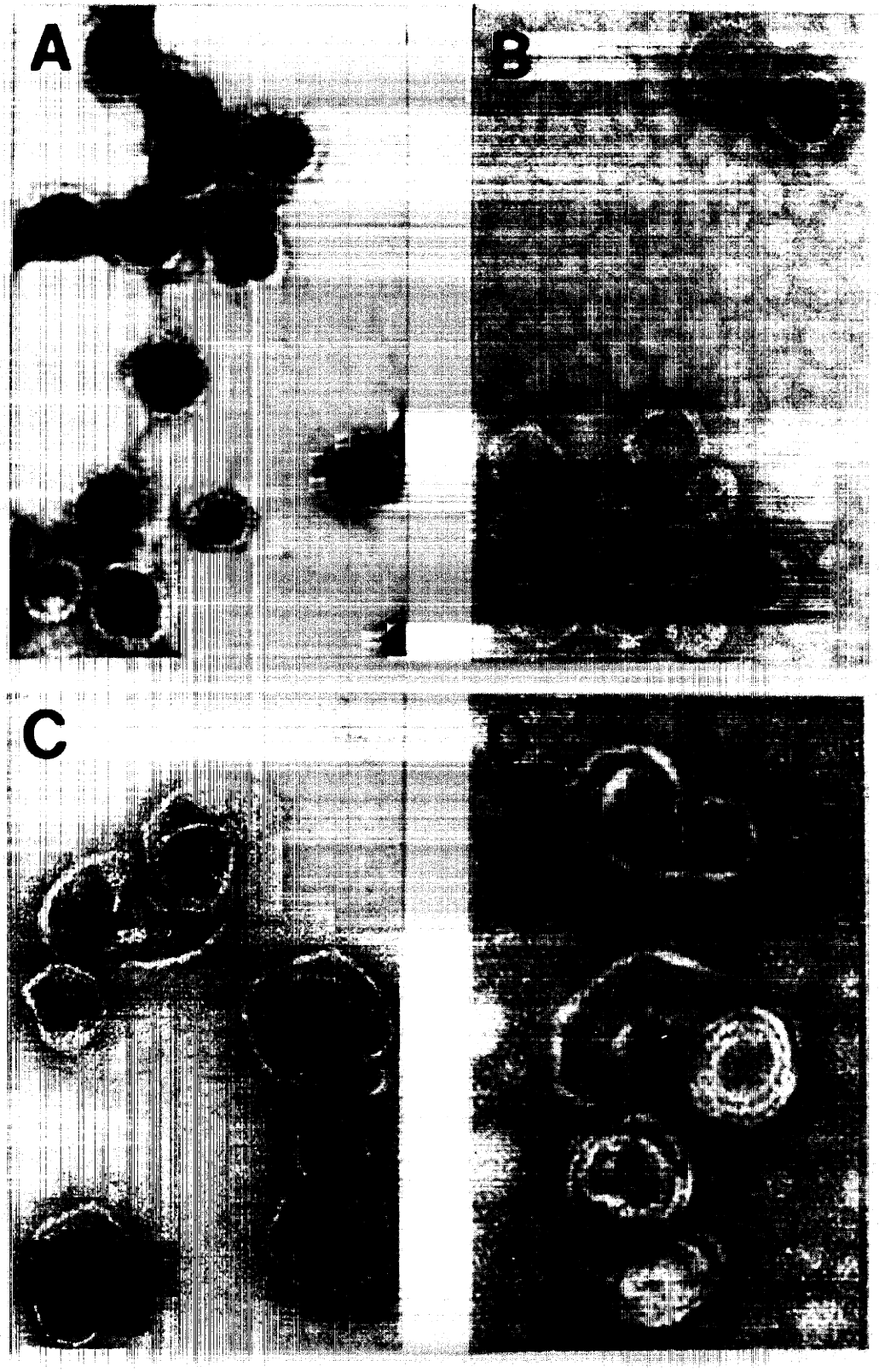

<--Spirals-->
c
<--Coat
<--Scaffolding
<--Spirals-->
240 S
d
<--Coat
<--Scaffolding
4
8
12
16
Bottom
240S
Top
Fraction Number

FIG. 2.8 Electron micrographs of the particles analysed in Fig. 2.7. 8a) $8^-$ shells, b) $8^-$ shells after incubation with scaffolding protein, c) spirals, d) spirals after incubation with scaffolding protein.

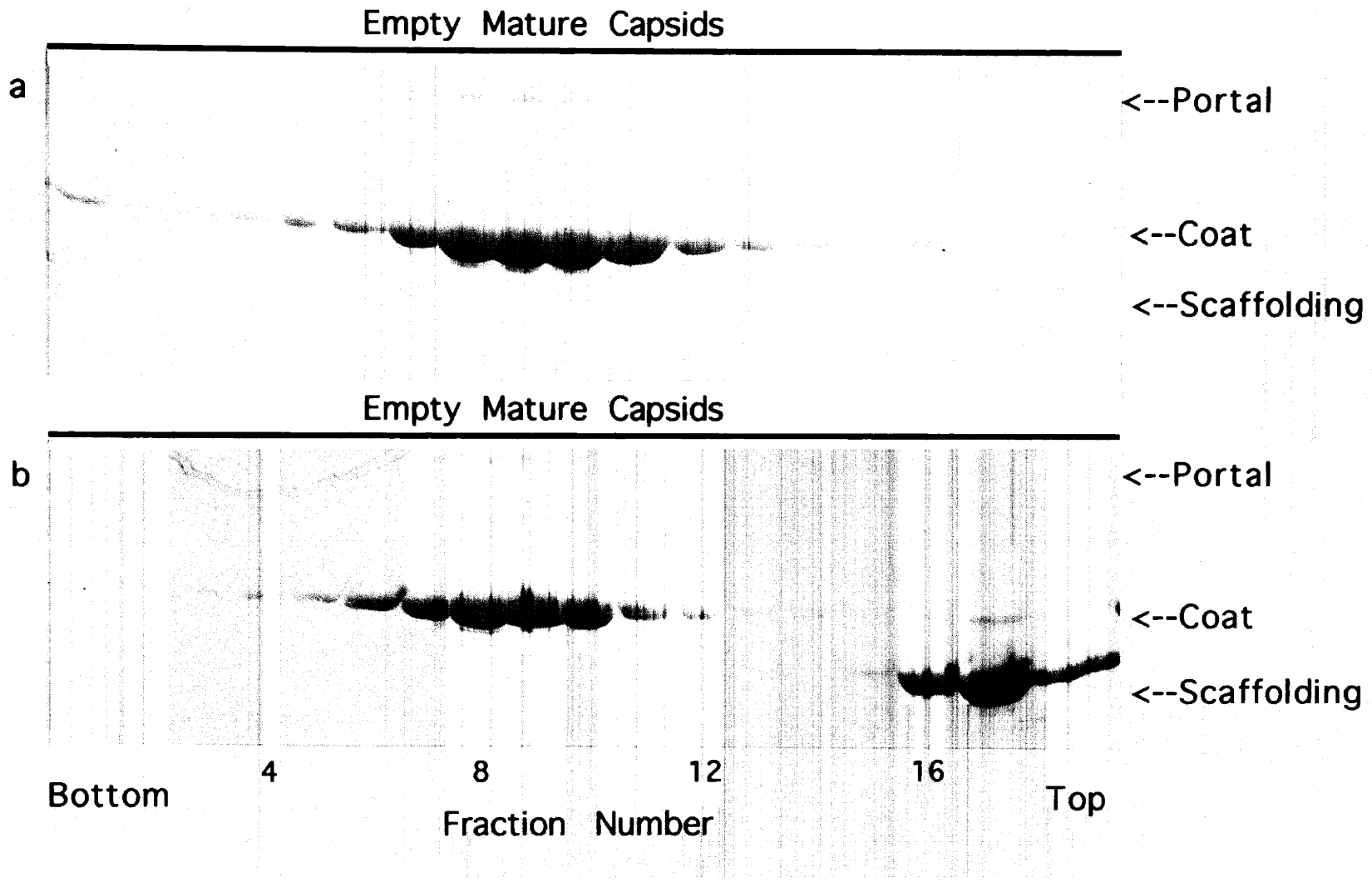

A
B
C

**Binding of scaffolding to capsids expanded *in vivo***

The maturation of the procapsid involves the release of all 300 scaffolding molecules (King and Casjens, 1974, Strauss and King, 1984). It is not clear whether this release is forced by conformational changes in the coat lattice or by the pressure of entering DNA. To test whether the mature capsid was capable of binding scaffolding subunits, empty mature heads were purifed from cells infected with phage mutant in a gene required for stabilization of DNA within the capsid (Strauss and King, 1984). These particles undergo DNA packaging, expansion and scaffolding release *in vivo*, but lose their DNA during the purification process.

Empty mature heads were incubated with scaffolding and analysed by sucrose gradient sedimentation and electron microscopy. As seen in Fig. 2.9, although scaffolding was present in excess, all of it sedimented as monomers, none being found associated with the capsid peak. Mature heads observed in the electron microscope appeared empty both before and after incubation with scaffolding (not shown). It appears that the mature heads, in contrast to spirals, do not bind the scaffolding protein at all.

FIG. 2.9 Binding of scaffolding to mature capsids expanded *in vivo*. Mature capsids at 1 mg/ml (a), or mature capsids following incubation overnight with scaffolding protein, both at 1 mg/ml (b), were centrifuged through 5-20% sucrose gradients. The gradients were fractionated and the fractions analysed by SDS-PAGE.


Empty Mature Capsids
a
<--Portal
<--Coat
<--Scaffolding
Empty Mature Capsids
b
<--Portal
<--Coat
<--Scaffolding
4
8
12
16
Bottom
Top
Fraction Number

## DISCUSSION

In viruses which assemble through a procapsid intermediate, the release of many scaffolding molecules is a critical step in virus maturation. While the scaffolding protein of bacteriophage Phi29 is recycled like that of P22 (Nelson et al., 1976), the phage T4 and lambda scaffolding molecules are cleaved and proteolytically degraded into small fragments (Onorato and Showe, 1975; Ray and Murialdo, 1975). It may be significant that the lambda procapsid structure, in contrast to P22, does not display channels through which an intact scaffolding protein might exit (Dokland and Murialdo, 1993). The herpes and adenovirus scaffolding equivalents are also proteolysed (Liu and Roizman, 1991; Hasson et al., 1992), but the herpes B capsid does display prominent channels through its caspsomeres (Baker et al., 1990). Whether any of these cleaved fragments are still capable of binding their capsids has not been investigated. Since the P22 scaffolding subunits exit intact and recycle, the complete molecules are both the precursor and product of the overall reaction.

### Reversible release of scaffolding subunits

The scaffolding release step can be reproduced *in vitro* for the procapsid of bacteriophage P22 by addition of low levels of GuHCl (Fuller and King, 1981). The effect of GuHCl appears to be due to its ability to denature proteins, as other, non-chaotropic salts do not have the same effect, although urea was also able to extract the scaffolding. The guanidine may be acting by denaturing a limited region of the scaffolding protein involved in mediating either binding to the coat lattice or binding between scaffolding subunits that stabilizes them within the procapsid. The low concentrations of GuHCl

required suggest that scaffolding is normally unstable or flexible in structure so as to allow it to be easily released upon DNA packaging.

Scaffolding protein can also be released from procapsids by heating, with the transition detected by calorimetry showing a midpoint at 48.5°C (Galisteo and King, 1993). The process had a very low enthalpy and was highly cooperative, suggesting that the disruption of the scaffolding/scaffolding interactions was key in the thermal process. The thermal loss of scaffolding subunits was also reversible.

That the *in vitro* extraction of scaffolding from the procapsids was completely reversible was unexpected, since it would appear entropically more favorable for 300 scaffolding molecules to remain free in solution at a concentration of 0.5 mg/ml, rather than tightly packing themselves into the procapsid shell, where the concentration of scaffolding is on the order of 100 times higher than in solution. One explanation is that the scaffolding binds rapidly and tightly to the capsid lattice upon reentry, so that the effective concentration of free scaffolding within the procapsid is always zero. The driving force for scaffolding reentry would be the concentration gradient, since the concentration of free scaffolding would always be lower inside the procapsids.

Scaffolding subunits were unable to bind to mature expanded heads indicating that expansion renders the scaffolding binding sites inaccessible, either because they are hidden by conformational changes in the coat protein or because closure of the holes leaves scaffolding unable to reach them. One function of the maturation conformational changes may be to prevent scaffolding from reentering the procapsid and interfering with DNA packaging.

**Two classes of scaffolding subunits exist within procapsids**

The binding of scaffolding appeared by kinetics to have two phases. Analysis of products on sucrose gradients suggested that half-filled capsids were an intermediate in the refilling process. This suggests the presence of two classes of scaffolding molecules in the reaction. Since the extraction of scaffolding from procapsids by bis-ANS is also a two-phase process (Teschke et al., 1993), the two classes of scaffolding molecules must exist within the procapsid, rather than being alternate solution conformations, one of which rearranges before entering the capsid. The two classes of scaffolding subunits may result from binding to two distinct sets of binding sites within the capsid, presumably respresenting different positions within the T=7 lattice. The other possibility is that a subset of the scaffolding molecules undergo conformational changes during the refilling process.

Since procapsids can assemble *in vitro* with a minimum of only half of the total number of scaffolding molecules found *in vivo* (Prevelige et al., 1988), we favor a model in which this population of scaffolding subunits occupies specific and rapidly filled sites on the coat lattice The additional molecules may occupy less specific positions, perhaps binding to the first set of scaffolding subunits rather than directly to the coat protein. Although not required for assembly, this second class of subunits may be needed to fill the remaining space in the capsid, so as to increase capsid stability or prevent the incorporation of extraneous proteins during assembly within the cell (Earnshaw and Casjens, 1980).

**Scaffolding functions**

The primary function of scaffolding protein is to insure assembly of coat protein into properly dimensioned T=7 capsids (Earnshaw and King,

1978). According to the Caspar and Klug (1962) quasi-equivalence model for virus structure, this means that scaffolding must insure the proper placement of 5-fold and 6-fold centers to form a closed lattice of coat molecules. Strong binding sites for scaffolding may only be generated at specific intersections of coat subunits within a T=7 icosahedral lattice. A similar model was proposed for the binding of assembly proteins (APs) to clathrin during the formation of coated pits (Keen, 1990). APs appear to have two different binding sites within clathrin cages, with the higher affinity site generated by a cluster of clathrin terminal domains formed at three-fold junctions in the assembled cage (Murphy and Keen, 1992).

If scaffolding protein binds to particular sites within the coat lattice, rather than simply to any large polymer of coat protein, then it should bind the minor class of empty shells produced in cells lacking scaffolding protein (Earnshaw and King, 1978). These are believed to have a lattice structure similar to that of complete procapsid shells. Scaffolding may not bind to spirals, which have their 5-fold and 6-fold vertices inserted in a non-directed mode.

That the properly sized $8^-$ shells are amply filled with scaffolding, while the spirals bind very little scaffolding, primarily in their centers where the curvature is closest to that of the procapsids, supports this model. If as our data suggest, only half of the approximately 300 scaffolding molecules bind to these specific sites, then there could be 140 molecules of scaffolding bound at the 3-fold centers, or 180 molecules bound as trimers at the 6-fold centers. Scaffolding protein may help coat protein assemble by binding to and stabilizing transient coat oligomers, in particular those oligomers which have adopted the appropriate conformations to fit within the T=7 procapsid lattice.

We cannot yet rule out the possibility that scaffolding protein does not bind efficiently to the spirals because the spiral coat lattices are in the expanded conformation. A temperature-sensitive mutation in the coat protein of bacteriophage T4 produces aberrant structures similar to spirals, that were expanded (Onorato et al., 1978). In this case, one function of scaffolding proteins might be to help stabilize coat subunits in assembly competent precursor states until the shell is properly closed.

Coat proteins bearing temperature-sensitive mutations form dimers and trimers when refolded *in vitro* at low temperature, in contrast to wild-type protein which remains monomeric (Teschke and King, 1995). When mixed with scaffolding protein, these oligomers do not assemble procapsids. These stable dimers and trimers might represent conformations found in the mature capsid, which has additional two-fold contacts compared to the procapsid (Prasad et al., 1993). The mutant coat proteins do form procapsids at low temperature *in vivo* (Gordon and King, 1993), where scaffolding protein is present and may forestall the formation of the non-assembly competent oliogomers.

The scaffolding protein may have functions in addition to morphogenesis. It has been shown that while empty mature capsids cannot package DNA *in vitro*, procapsids can (Poteete et al., 1979), although whether the procapsids must contain scaffolding to be active is not known. As the products of the *8am* infection demonstrate, coat protein can assemble into procapsid-like shells in the absence of scaffolding protein, although at lower efficiency; this alternative pathway may be used at low levels during wild-type infections. If scaffolding protein does play a role in DNA packaging, then the ability of scaffolding subunits to enter empty procapsids could serve an

important function *in vivo*, by restoring the ability of these capsids to package DNA.

Scaffoldings are not the only proteins which interact with viral capsid lattices. The T4 soc protein and the lambda D protein bind at the three-fold centers of symmetry on the outer surface of the mature capsid, after DNA packaging and expansion (Steven et al., 1992; Dokland and Murialdo, 1993). These proteins appear to help stabilize the mature virion; addition of the soc protein increases the temperature required for thermal denaturation of T4 by 5°C (Steven et al., 1992). Scaffolding differs from these molecules in binding to the inside of the precursor rather than the outside of the mature form, and in that its binding is not permanent but reversible upon DNA packaging. Perhaps scaffolding proteins help to stabilise the precursor capsid against expansion to the energetically favored mature form until after DNA packaging begins. The study of scaffolding/capsid interactions may provide new insights into the subject of protein binding to regular lattice structures.

# CHAPTER 3

# CHARACTERIZATION OF ASSEMBLY MUTANTS IN THE BACTERIOPHAGE P22 SCAFFOLDING PROTEIN

## INTRODUCTION

The first product of assembly of the double-stranded DNA phages (Casjens and Hendrix, 1988) as well as the herpesviruses (Sherman and Bachenheimer, 1988; Lee et al., 1988) and adenoviruses (D'Halluin et al., 1978; Edvardsson et al., 1976) is a precursor capsid that serves as a substrate for DNA packaging. Formation of these precursors requires large numbers of scaffolding molecules, proteins that are not found in the mature virions. Upon the commencement of DNA packaging, all the scaffolding molecules exit the capsid, being either proteolysed into small fragments or released intact. In the absence of scaffolding the coat proteins of dsDNA phages (Hendrix, 1985; Casjens and Hendrix, 1988) and herpesvirus (Desai et al., 1994; Matusick-Kumar et al., 1994; Thomsen et al., 1994; Tatman et al., 1994) form aberrant structures, demonstrating that scaffolding proteins play an essential role in morphogenesis. One might imagine that the scaffolding serves simply as a core, around which the coat subunits can polymerize to form a closed shell of the correct size to contain the viral genome. If this were so, scaffolding proteins could be quite simple molecules — essentially support rods with sticky ends, one to bind the coat protein and one to join the scaffolding molecules together to form a core.

The actual role of the scaffolding appears to be significantly more complex. The assembly pathway of the bacteriophage P22 procapsid which has

been studied in *in vitro* does not begin with the assembly of a scaffolding core (Prevelige et al., 1988). Instead, assembly appears to proceed by the serial addition of both coat and scaffolding subunits onto the edges of a growing shell (Prevelige et al., 1993b). While the scaffolding proteins of T4 (Traub and Maeder, 1984; van Driel and Couture, 1978b) and herpesvirus (Newcomb and Brown, 1991; Kennard et al. 1995) are capable of forming naked cores, the *in vivo* pathway of these viruses is believed to also involve copolymerization (Kellenberger, 1990; Thomsen et al., 1995). This implies that the scaffolding proteins have a more active role in the assembly of viral coat subunits into procapsids, one that may involve concerted conformational changes in both coat and scaffolding proteins.

Assembly of a functional procapsid requires more than correct polymerization of the coat protein. As shown in Fig. 3.1, P22 procapsids contain minor components in addition to the coat and scaffolding proteins. The portal protein, present in 12 copies per procapsid (Bazinet et al., 1988), comprises the channel through which the DNA is packaged. The portal complex is located at one 5-fold vertex of the procapsid and serves as the site for tail attachment in the mature phage (Hartweig et al., 1986). The procapsid also includes three pilot proteins required for DNA injection into the host cell (Botstein et al., 1973; Hoffman and Levine, 1975a, b). Their location within the capsid is not known, althought their low copy number, 10 - 20 per procapsid (Casjens and King, 1974), implies that they can only be present at a few specialized positions. They might all be located at the portal vertex, or in one or two copies at each vertex (Thomas and Prevelige, 1991). Incorporation of these proteins is thought to be an early step in assembly, as neither the portal (Poteete et al., 1979; Fuller and King, 1982) nor the pilot proteins (Jarvik

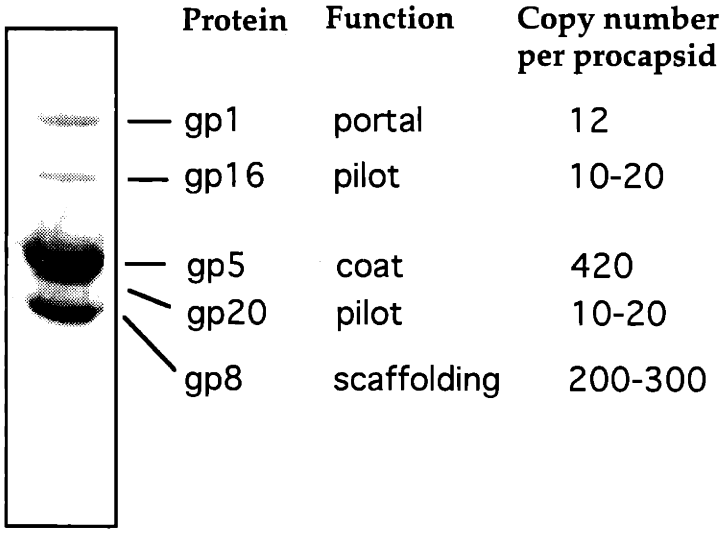



Figure 3.1a Protein composition of P22 procapsids

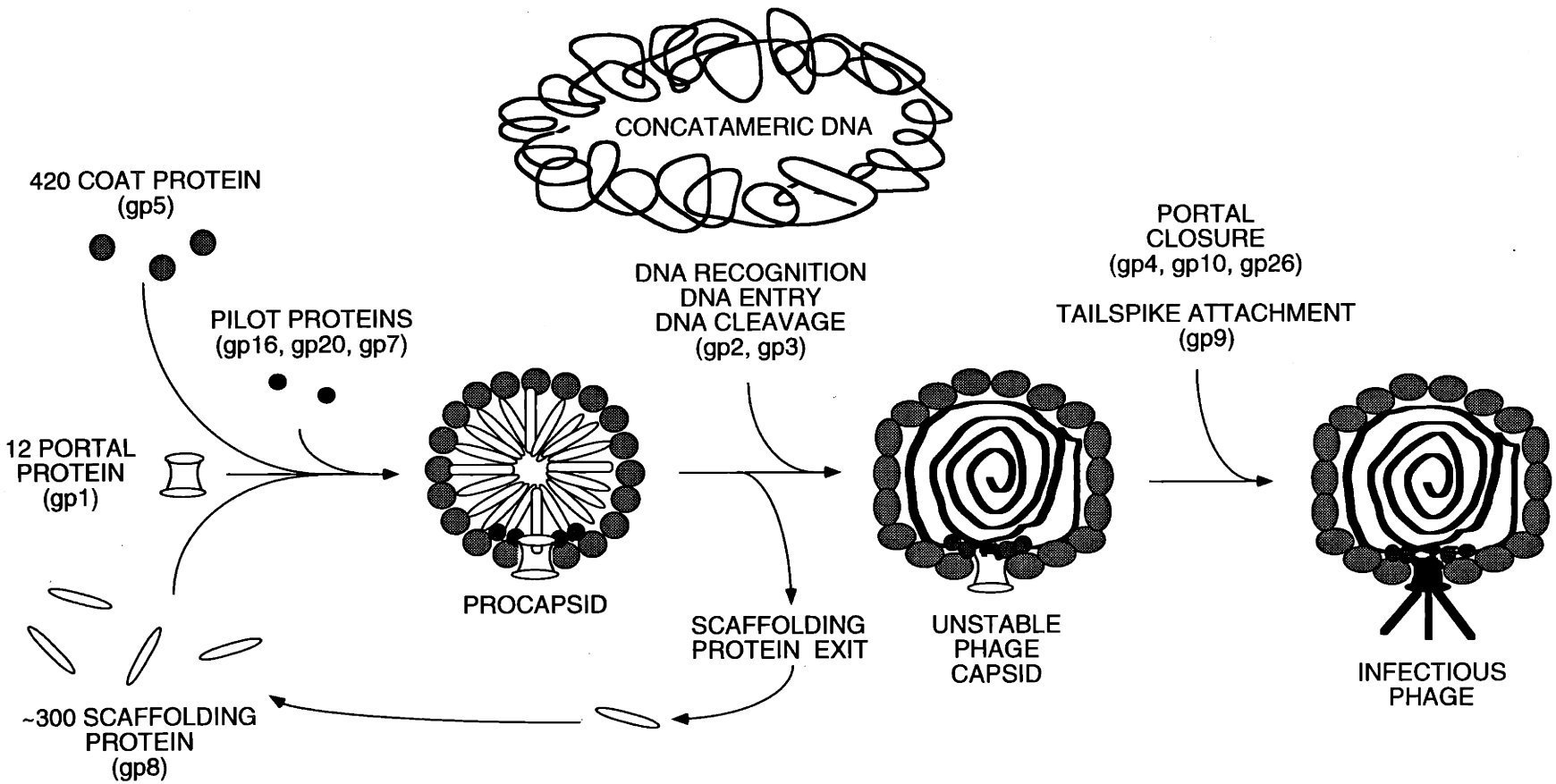



FIG. 3.1b Assembly pathway of bacteriophage P22

and Botstein, 1973; Thomas and Prevelige, 1991) can be added onto completed procapsids.

Incorporation of these minor proteins may be an additional function of the scaffolding protein. Structures formed by phage coat proteins in the absence of scaffolding lack portals and other minor proteins (Earnshaw and King, 1978; Ray and Murialdo, 1975; van Driel and Couture, 1978a). Genetic evidence from bacteriophage lambda indicates that the presence of the lambda scaffolding protein is required for the formation of the dodecameric portal ring, the first step in procapsid assembly (Murialdo and Becker, 1978; Murialdo, 1979; Kochan and Murialdo, 1983). A role for scaffoldings in procapsid initiation and portal insertion implies that scaffolding molecules must possess sites for interaction with portal protein in addition to coat and other scaffolding molecules. This idea also suggests that scaffolding molecules may play different roles at different times during the assembly process.

Another distinct process in capsid assembly is capsid maturation, during which the scaffolding is released, the DNA is packaged, and the coat protein undergoes conformational changes resulting in expansion and angularization of the capsid (Prasad et al., 1993). The signalling pathway for initiation of this lattice expansion has not been defined. The conformational changes in the coat protein may be induced through interactions with the portal, with the exiting scaffolding, or by direct interactions with the entering DNA.

In order to exit, the scaffolding must cease its binding to the coat protein, suggesting that the scaffolding molecules must at least undergo some sort of conformational change to be released from the procapsid. While this release can be induced *in vitro* by the denaturant GuHCl, as described in Chapter 2, this cannot be the mechanism used within the cell. When DNA

packaging is blocked by mutations in the DNA packaging proteins, the cell accumulates unexpanded procapsids which still contain scaffolding (King et al., 1973). Perhaps the scaffolding protein can sense the entry of DNA; if so, an additional interaction site with DNA might exist.

It has proven difficult to study these processes *in vivo*, since the steps in phage assembly are rapid and tightly coupled. The order of events involved in phage maturation is still uncertain. To help elucidate these functions, I set out to isolate mutants in the scaffolding gene and determine their effects on scaffolding function *in vivo*. In this way, I hoped to selectively perturb specific functions of the scaffolding protein at different points along the assembly pathway, and thus generate a more detailed picture of the assembly process. Moreover, by determining the defects produced by mutants at different positions, it would be possible to understand which regions of the molecule might be involved in different functions.

## MATERIALS AND METHODS

### Bacteria

All cell lines used were derivatives of *Salmonella typhymurium* LT2. The suppressor minus host DB7136 (leu amA141, his amC525) and the two supressor plus derivatives DB7155 supE20(Gln) and DB7156 supF20(Tyr) that were used to propagate amber mutants are described by Winston et al., (1979). Cells used for the GroE rescue experiments were DB7136 cells carrying a plasmid with the *E. coli* GroE gene (pOF39) or a control plasmid (pBR322). (Gordon et al., 1994).

### Phage

The isolation of mutants 8tsN102, 8csRN26D, 5cs577 and 5cs567 has been described by Jarvik and Botstein (1975). Other mutants used were 8amN123 and 8amN26 (Botstein, Chan and Waddell, 1972). The U100 mutant series, including cs and ts mutants, and the U200 series amber mutants were isolated in the lab of J. King.

All phage used in these experiments carried the mutation $c_{1\text{-}7}$, which prevents lysogeny. Some also carried the mutations 13amH101, which delays lysis, and 2amH202 or 3amN6, either of which prevents DNA packaging (King et al., 1973).

### Media

LB medium was used to grow bacteria for plating experiments, complementation tests and preparation of phage stocks. LB plus 100 μg/ml ampicillin was used for growth and plating experiments with the plasmid containing strains. Superbroth (Fuller and King, 1981) was used to grow

bacteria for the preparation of infected cell lysates and procapsid preparations. Dilution fluid (DF) used for serial dilutions of phage for titering was 0.1% tryptone, 0.7% NaCl, 2mM $MgSO_4$.

**Isolation of new mutants and crosses**

Revertants of gene 8 amber mutants were selected by plating on the non-supressing host at 30°C. These revertants were screened by stabbing onto plates incubated in parallel at 17°C, 30°C and 39°C for plaques that could not grow at high or low temperature. Revertants and supressors of temperature and cold-sensitive mutations were selected by plating at 39°C or 17°C respectively, and screening the resultant plaques for inability to grow at the opposite temperature. Plaques displaying ts or cs phenotypes were grown up into stocks (Gordon and King, 1994) and retested. The mutations were assigned to gene 8 or gene 5 by liquid complementation tests. Those in gene 8 were mapped to one of four regions AA, BB, CC, or DD within the gene by recombination with mapping plasmids containing different P22 gene fragments as described by Casjens et al. (1991). To cross the new mutants into amber backgrounds, exponentially growing DB7155 cells in LB at a concentration of $2x10^8$/ml were infected with phage strains carrying desired alleles, each at a multiplicity of infection (MOI) of 5 phage per cell. Progeny phage were screened for desired alleles.

**Single burst experiments and complementation experiments**

A portion of an overnight culture of 7136 cells was diluted 1/100 in LB and grown to $4x10^8$ cells/ml. Each infection contained 0.2 ml of these cells and an equal volume of phage at a concentration of $4x10^9$/ml, for an MOI of 10. In the mixed infections, each phage contributed half of the total titer. Infections

were allowed to proceed for 2 hours at 30°C or 40°C, and 5 1/2 hours at 17°C, then terminated by dilution into DF saturated with chloroform. Resultant bursts were determined by plating at 30°C.

**DNA Sequencing**

DNA sequencing was performed as described in Gordon and King, 1993. Briefly, a 1.5 kB region of phage DNA was amplified by symmetric PCR using primer 1, corresponding to nucleotides 3830–3854 and primer 2, complimentary to nucleotides 5305–5276. The numbering is according to the notation of Eppler et al., 1991, in which the scaffolding coding region consists of nucleotides 3893–4801. The double-stranded DNA product was purified on an agarose gel, and the bands cut out and cleaned using GeneClean (Bio101). This DNA was used as a template for synthesis of single stranded DNA by asymmetric PCR using one or the other of the original primers. After purification with GeneClean, this DNA was used as the template for dideoxy DNA sequencing using the Sequenase kit (U. S. Biochemical). Primers used for sequencing were primer 3 (corresponding to nucleotides 4299–4315) and primer 4 (complimentary to nucleotides 4426–4410) for sequencing region BB; primer 5 (corresponding to nucleotides 4675–4699) and primer 6 (complimentary to nucleotides 4751-4727) for region CC. All primers were synthesized by the MIT Biopolymers Laboratory.

**Preparation of cell lysates**

7136 cells were grown to $4x10^8$/ml in superbroth. 20 ml tubes were each infected with a phage strain at an MOI of 10. The infections were allowed to proceed for 3 hours at 30°C or 39.5°C, and for 5 1/2 hours at 17°C. Cells were then concentrated by low speed centrifugation and resuspended to 0.5 ml in

buffer B (50 mM Tris; 25 mM NaCl; 2 mM EDTA, pH 7.6). The cells were lysed by three cycles of freezing and thawing. Lysates were cleared of DNA by addition of 20 mM $MgS0_4$ and 10 μg/ml DNAse (Sigma).

Samples (200 μl) of lysates were loaded onto 5 ml 5-20% sucrose gradients, with 0.2 ml cushions of 50% CsCl in 20% sucrose. The gradients were centrifuged for 35 minutes at 35,000 rpm in a Beckman SW50.1 rotor. Gradients were fractionated through a pinhole in the bottom of the tube into 18 fractions. Fractions were concentrated by TCA precipitation, then analyzed by SDS-PAGE.

**Electron microscopy**

Samples of cell lysates diluted to approximately 1 mg/ml were deposited onto carbon-coated copper grids, negatively stained by 2% uranyl acetate, and air dried. Grids were examined in a JEOL 1200 electron microscope at 80 kV. At least four micrographs were taken of each sample, and 150–250 particles were counted. The structures observed were classified into the following categories: Phage – electron-dense particles with an angular shape that usually have a tail at one corner. Procapsids – round particles with rough edges. These may have some internal density or appear empty; I did not try to count seperate classes of full versus empty procapsids. Empty heads – angular particles with smoother, thinner edges than procapsids and no internal density. Small – round particles that are approximately half the diameter of procapsids. Aberrant – spirals, figure 9s, and oversized or misshapen capsids.

**Agarose gels**

Samples of lysates or purified capsids were expanded *in vitro* by heating at 65°C for 20 minutes (Galisteo and King, 1993). Expanded and unexpanded capsids were separated by electrophoresis on 1.8% agarose gels as described in Galisteo and King (1993).

**Scaffolding Extraction**

Procapsids made by cells infected with mutant phage at the permissive temperature were purified as previously described (Prevelige et al., 1988). Samples at 1.5 mg/ml were incubated overnight in varied concentrations of guanidine hydrochloride at 25°C. The amount of scaffolding extracted at each concentration was quantified by separating free scaffolding from capsids by sucrose gradient sedimentation as described in Chapter 2, except that only 6 fractions rather than 18 were collected from each gradient.

**Isolation and sequencing of new mutants in gene *8***

Previous mutagenesis of the P22 genome over the course of two decades had resulted in the production of only two ts mutants mapping to gene 8, alleles N102 and U172. Both of these mapped to the same location and were found to cause identical alterations in the DNA sequence. (M. Galisteo, unpublished results). By comparision, the same mutant searches had yielded over 20 ts alleles in the coat protein, gp5 and over 50 in the tailspike, gp9, raising the possibility that the scaffolding protein was less susceptible to ts lesions, perhaps because most of its sequence was acting as a simple structural support rod. Jarvik and Botstein (1975), found that selecting pseudorevertants or suppressors of existing conditional lethal mutations was an efficient way to find new ts and cs mutants. The only known cs mutant in gene 8 had been selected as a psuedorevertant of a gene 8 amber (Jarvik and Botstein, 1975). I adopted this strategy to select additional ts or cs mutants in gene 8.

Many of the starting amber mutants did not yield any ts or cs pseudo-revertants (Table 3.1), perhaps because all substitutions at these sites are either permissive or lethal. Two amber sites did yield ts pseudorevertants, one at the site of the original cs pseudorevertant; this mutation had both cs and ts phenotypes. I also attempted to select gene 8 mutants as supressors of mutations in gene 5, on the assumption that the coat and scaffolding proteins must interact. Although there are many ts coat alleles, all have the phenotype of forming inclusion bodies before interacting with scaffolding (Gordon and King, 1993). They did not seem likely candidates to be suppressed by scaffolding mutants. Instead I sought suppressors of three coat cs mutations, which there was reason to believe might affect coat/scaffolding interactions.

TABLE 3.1
Mutants tested for new pseudorevertants or second-site suppressor mutations

| Original Mutant | Site of Mutation | Number of plaques screened | New mutants obtained |
|---|---|---|---|
| 8amU237 | unknown | 175 | none |
| 8amU238 | L226 | 200 | none |
| 8amU239 | Q8 | 75 | none |
| 8amU240 | Y214 | 200 | 8tsY214W |
| 8amU241 | Q154 | 150 | none |
| 8amH202 | Q154 | 150 | none |
| 8amN26 | Q149 | 100 | 8ts/csQ149W |
| 5cs567 | T10I | 100 | none |
| 5cs577 | N414S | 100 | none |
| 5csU102 | R101C | 100 | 8tsL177I 5ts |
| 8tsN102 | S242F | 100 | none |
| 8tsY214W | Y214W | 100 | none |

The mutation N414S causes production of procapsids that do not package DNA and are blocked in expansion *in vitro* (Gordon, Lee, Reiner and King, unpublished results). The two N-terminal mutations result in procapsids that have lost most of their scaffolding. (Gordon, Lee, Reiner and King; Greene and King, unpublished results). Only the coat cs mutant R101C yielded a second site suppressor in gene 8 with a ts phenotype. The new strain bearing the suppressor was crossed to an amber in gene 5 and backcrossed to wild-type so as to eliminate the parent cs mutation.

The mutants were assigned to gene 8 by liquid complementation tests with both gene 5 and gene 8 amber mutants, then mapped to specific regions of gene 8 by recombination with both gene 8 ambers at known locations and

with mapping plasmids carrying pieces of the P22 genome (Casjens et al., 1991). Regions of gene 8 containing the new mutations as well as the original amber and cs 5 mutants were sequenced to determine the amino acid changes. The results are shown in Table 3.2.

There are five mutants at four different sites (Fig. 3.2). The location of the changes are consistent with the locations of the parent amber mutations and with the genetic mapping. The strains with mutations Q149Y and Q149W also carry the mutation A199V. Since this change is also present in the parent amber strain, which is neither ts nor cs on the Gln-inserting supressor host, this appears to be a silent mutation. In most cases the change results in the substitution of a bulky aromatic residue for a smaller sidechain which may result in destabilization of the protein fold. The one exception, L177I, is a more conservative substitution, but the beta-branched residues such as isoleucine are known to destabilize alpha helices (Cornish et al., 1994).


FIG. 3.2 Location of conditional lethal mutations within the bacteriophage P22 scaffolding gene.

TABLE 3.2

SEQUENCE CHANGES IN TEMPERATURE AND COLD-SENSITIVE MUTANTS IN THE P22 SCAFFOLDING GENE

| Mutant | Nucleotides sequenced | nucleotide substitution | amino acid substitution | Local amino acid sequence |
|---|---|---|---|---|
| 8ts N102 | 505-800 | C765T | Ser242--> Phe | ELTRL**S**ERLTL |
| 8ts pseuodorevertant of 8amU240 | 465-680 | A641G<br>T642G | Tyr214--> Trp | SAALM**Y**HLGAN |
| 8ts suppressor of 5csU102 | 505-800 | C529A | Leu177--> Ile | DAAEK**L**NIPDY |
| 8ts/cs pseudorevertant of 8amN26 | 355-665 | C445T<br>A446G<br>C596T | Gln149--> Trp<br><br>Ala199--> Val | NAVAE**Q**GRKTQ |
| 8cs pseudorevertant of 8amN26 | 335-800 | C445T<br>G447T<br>C596T | Gln149--> Tyr<br><br>Ala199--> Val | NAVAE**Q**GRKTQ |
| 8amU240 | 505-800 | T642G | Tyr214--> amber | |
| 8amN26 | 225-665 | C445T<br>C596T | Gln149--> amber<br>Ala199--> Val | |
| 5csU102 | 215-485 | C301T | Arg101--> Cys | DETAY**R**RRIQS |

**Phenotypes of mutants *in vivo***

In order to determine the effects of these mutations on the phage assembly pathway, cells were infected with mutant strains at both permissive and non-permissive temperatures. The resulting lysates were clarified and concentrated. Lysates were examined by electron microscopy, to observe any large structures accumulated, and by sucrose gradient sedimentation and SDS-PAGE, to determine the protein composition of these structures. This approach assumes that some structures will in fact be made, but this assumption seemed reasonable given that even in the absence of scaffolding protein, as in a gene 8 amber infection, the coat subunits can form some aberrant large assemblies (King et al., 1973; Earnshaw and King, 1978).

At the permissive temperature of 30°C, all the mutants were similar to wild-type, producing both procapsids and infectious phage. At the non-permissive temperature, all the mutants were capable of assembling with coat protein into large structures, visible in the micrographs and as rapidly sedimenting peaks on the sucrose gradients. The amount of coat protein assembled into structures appeared roughly comparable to wild-type, indicating that the mutants had not lost the ability to associate with the coat protein. However, the capsids produced by each mutant showed specific defects.

At 40°C, the wild-type strain produced procapsids and infectious phage (Fig. 3.4a). The phage are the more angular, electron-dense particles with tails. The procapsids are rounder with some stain penetrating the shell. The sucrose gradient revealed a peak at the bottom, of the heavy, DNA-containing phage, and another around fractions 4-6, containing the procapsids (Fig. 3.3a). The procapsid peak includes five proteins: coat, scaffolding, portal, and the

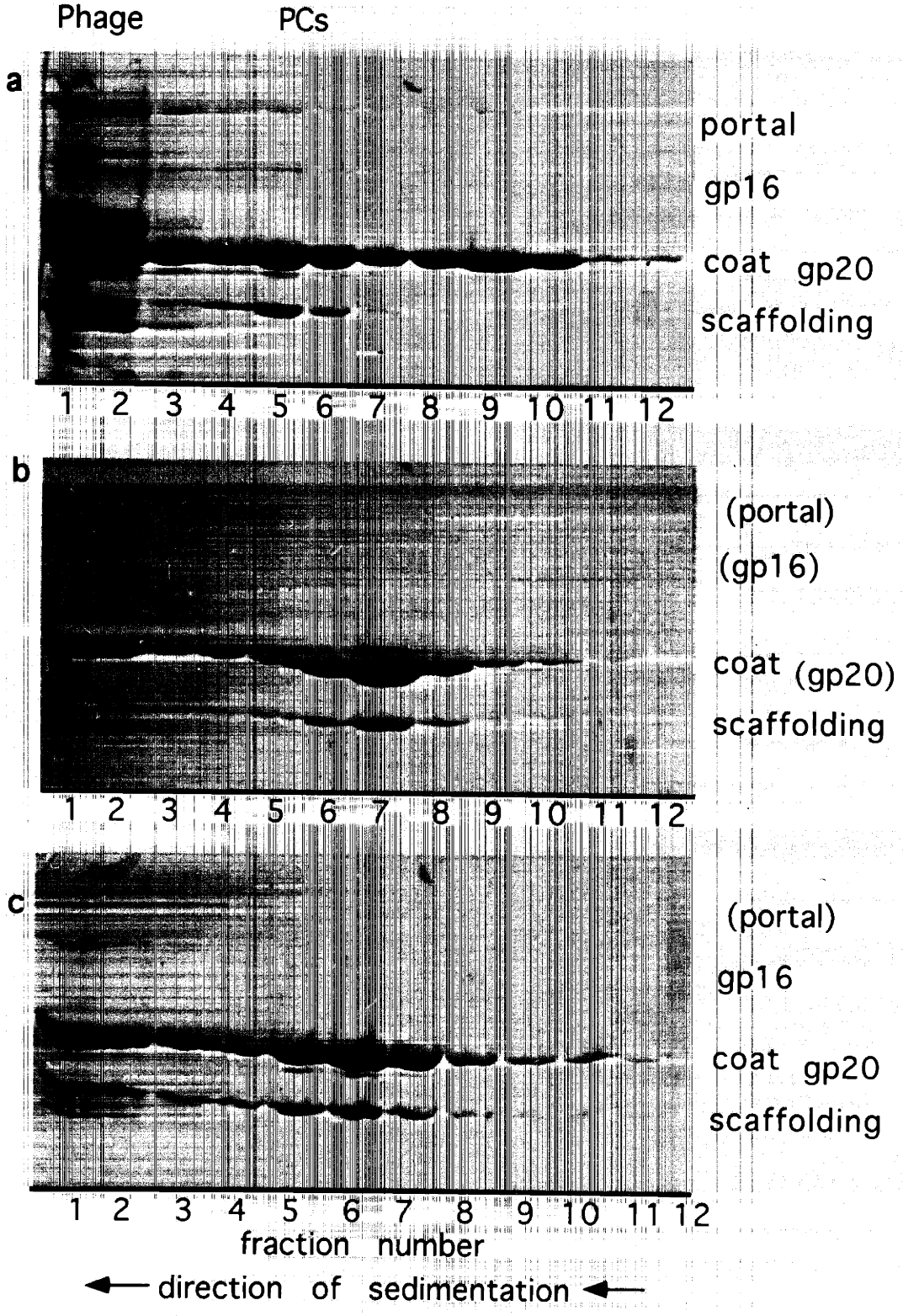
two pilot proteins gp16 and gp20. The third pilot protein gp7 is too small to be detected on these gels.

None of the ts mutants produced any infectious phage, as their burst sizes were reduced by over 100-fold (Table 3.3). The mutant S242F has been previously characterized by Bazinet and King (1988). Consistent with their results, this mutant produced procapsids that contained only coat and scaffolding. These structures failed to include the portal or the products of genes 16 and 20. These procapsids also included less than the usual amount of scaffolding protein and were therefore somewhat lighter, sedimenting more slowly on the sucrose gradient than wild-type procapsids (Fig 3.3b). The procapsids produced by this mutant appeared morphologically normal (Fig. 3.4b).

The mutant Y214W had a similar phenotype, also yielding morphologically normal procapsids that lacked the portal. However, these capsids did contain the usual amounts of gp16 and 20, and appeared to have the full complement of scaffolding protein, thus sedimenting slightly faster than the S242F procapsids (Fig. 3.3c).

The mutant L177I accumulated procapsids that lacked only gp 16 (Fig. 3.3d). This mutant lysate also contained many particles which resemble mature, tailed phage (Fig. 3.4d). These particles were not infectious, since the number of plaque-forming units in this lysate was reduced a thousand-fold compared to wild-type. Presumably, the phage also lack gp16 and are thus incapable of infection. The amount of phage produced, however, was much less than in the wild-type infection. While the cells infected with the wild-type strain were filled primarily with mature phage at the end of the infection (Fig. 3.7) those infected with the mutant L177I still contained mostly procapsids, suggesting that this mutant caused an inhibition of maturation.

FIG. 3.3 Sucrose gradients of lysates from cells infected with temperature-sensitive scaffolding mutants at 40°C. Samples (200 µl) of lysates prepared as described in Materials and Methods were centrifuged through 5-20% sucrose gradients. 18 fractions were collected from each gradient and their protein composition analysed by SDS-PAGE. The positions to which wild-type phage and procapsids sediment on the gradient are indicated at the top of the figure. Procapsid proteins not present within a given gradient are marked with brackets. Structures made in the scaffolding amber infection, shown as a control, contain only coat protein. (a) wild-type, (b) 8ts S242F, (c) 8ts Y214W, (d) 8ts L177I, (e) 8ts/cs Q149W, (f) 8 amber.


Phage
PCs
a
portal
gp16
coat gp20
scaffolding
1 2 3 4 5 6 7 8 9 10 11 12
b
(portal)
(gp16)
coat (gp20)
scaffolding
1 2 3 4 5 6 7 8 9 10 11 12
c
(portal)
gp16
coat gp20
scaffolding
1 2 3 4 5 6 7 8 9 10 11 12
fraction number
direction of sedimentation

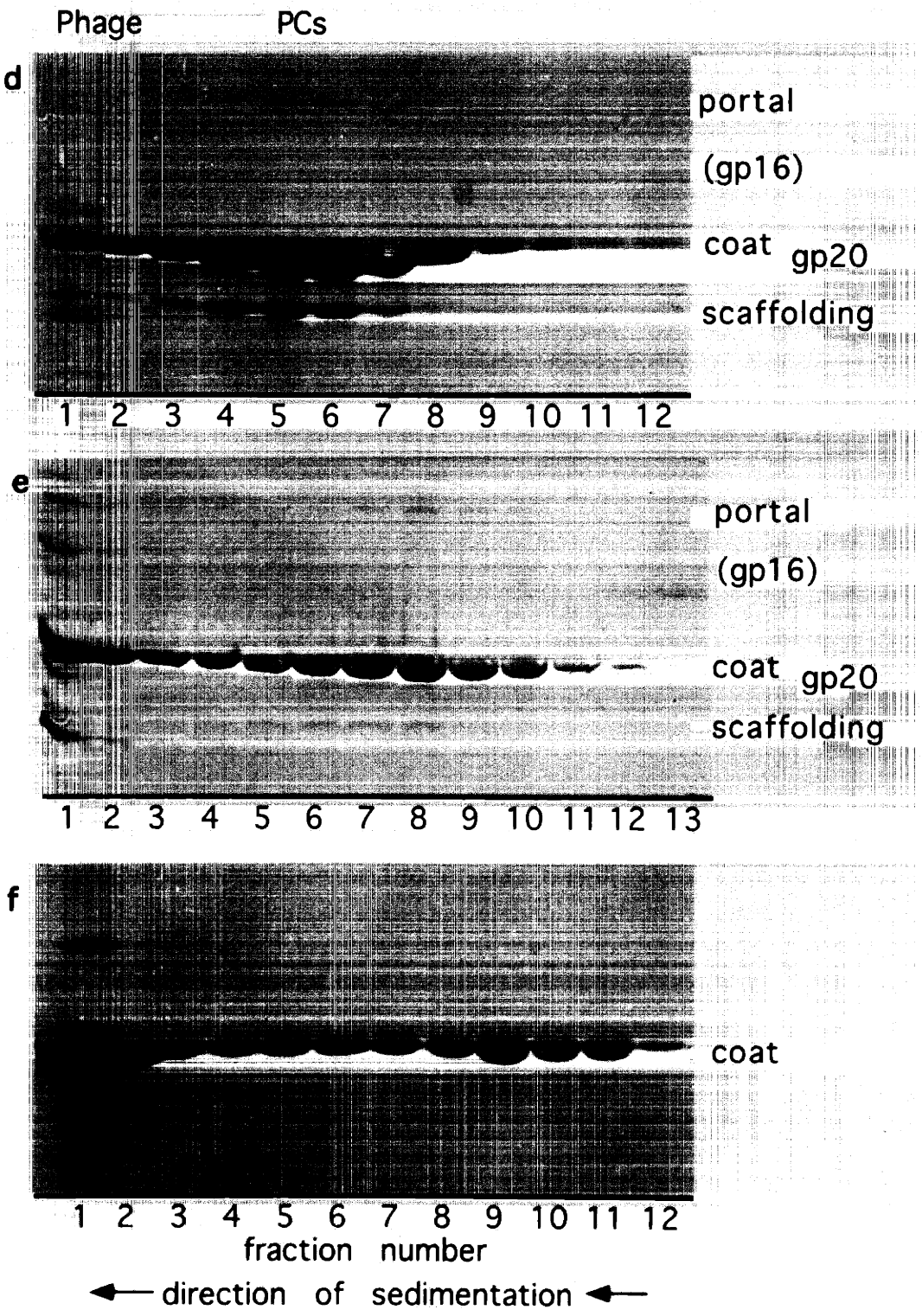
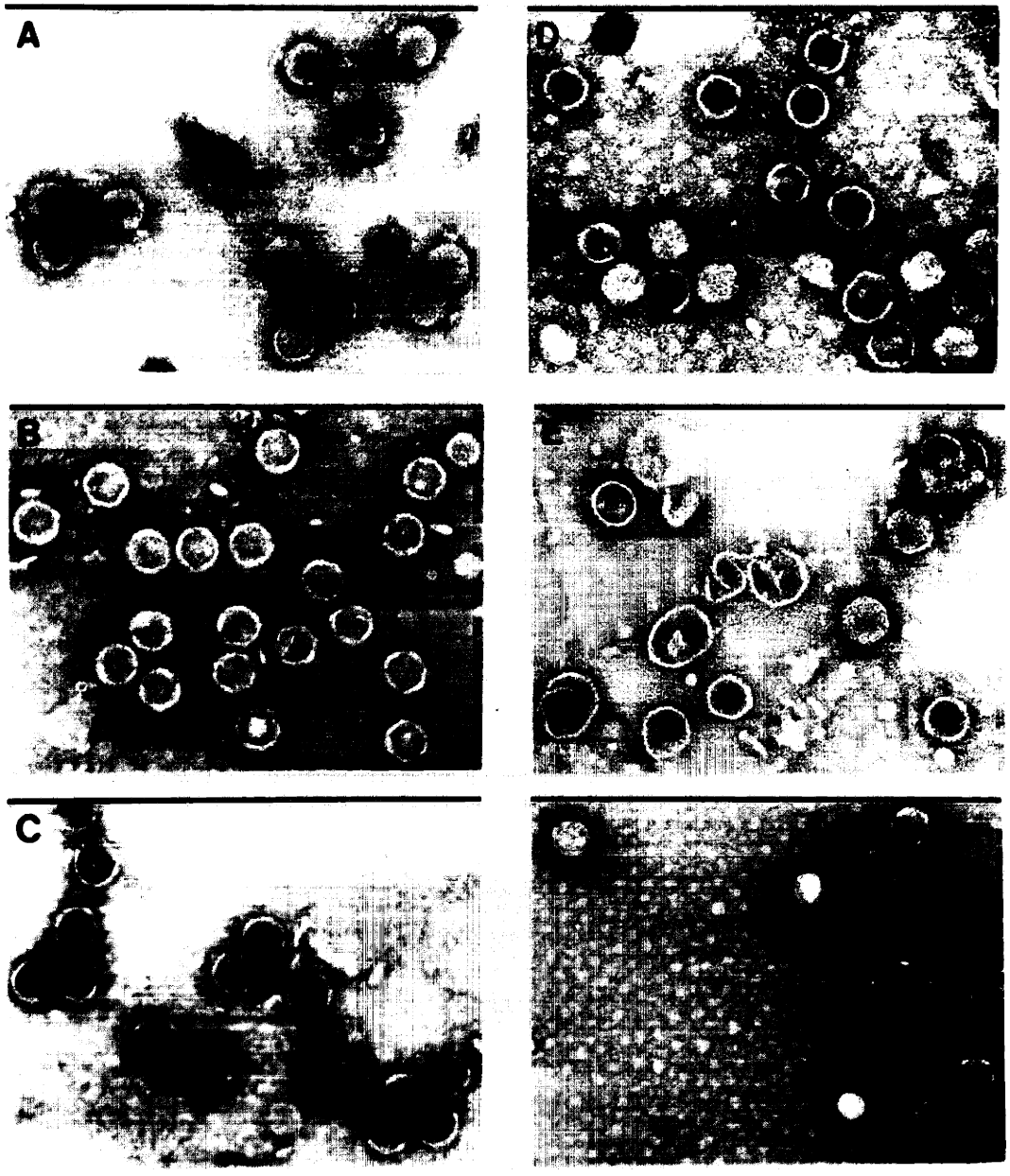

Phage
PCs
d
portal
(gp16)
coat
gp20
scaffolding
1 2 3 4 5 6 7 8 9 10 11 12
e
portal
(gp16)
coat
gp20
scaffolding
1 2 3 4 5 6 7 8 9 10 11 12 13
f
coat
1 2 3 4 5 6 7 8 9 10 11 12
fraction number
direction of sedimentation

FIG. 3.4 Electron microscopy of structures produced by gene 8 ts mutants at 40°C. The lysates subjected to sucrose gradient centrifugation in Fig. 3.3 were observed by negative stain at a magnification of 60,000x. (a) wild-type, (b) 8tsS242F, (c) 8ts Y214W, (d) 8ts L177I, (e) 8ts/cs Q149W, (f) 8 amber.


A
D
B
C

Mutant Q149W is both temperature and cold-sensitive. At high temperature (40°C), this mutant produced some normal appearing procapsids, but also many that were incorrectly formed. As seen in Fig. 3.4e, these particles appeared distorted in shape rather than round. The morphological defect produced particles both longer and wider than usual. Some of these structures were not closed, but resembled "figure 9s". This phenotype was different from that produced in the absence of scaffolding protein, as in a gene 8 amber infection. The structures produced in the 8 amber infection were far more severely aberrant spirals containing multiple whorls (Fig. 3.4f). Also, the 8 amber infection produced round particles smaller in size than procapsids, which were not seen in the Q149W lysate (Fig. 3.7). In addition, while the structures produced in the 8 amber infection contain only coat protein, those produced by Q149W appeared to contain portal protein, which is visible across the gradient in proportion to the coat protein present (Fig. 3.3e,f). The Q149W lysate also contained some non-infectious phage particles. As with L177I, the amount of phage produced was lower than for wild-type (Fig. 3.7).

Both substitutions at Q149 exhibited a cold-sensitive phenotype, although this was not as strict a defect as the ts phenotype; the burst size was reduced only by a factor of ten at 17°C. Both substitutions had a similar phenotype: procapsids of normal dimensions were produced, but many lacked scaffolding protein, as seen on the sucrose gradients (Fig. 3.5) and by their empty appearance in the electron micrographs (Fig. 3.6). The empty capsids did not appear to have undergone expansion, but displayed the round shape and rough edges typical of unexpanded procapsids.

FIG. 3.5 Sucrose gradients of lysates from cells infected with cold-sensitive scaffolding mutants at 17°C. Samples (200 µl) of lysates prepared as described in Materials and Methods were centrifuged through 5-20% sucrose gradients. 18 fractions were collected from each gradient and their protein composition analysed by SDS-PAGE. The positions to which wild-type phage and procapsids sediment on the gradient are indicated at the top of the figure. (a) wild-type, (b) 8ts/cs Q149W, (c) 8cs Q149Y.

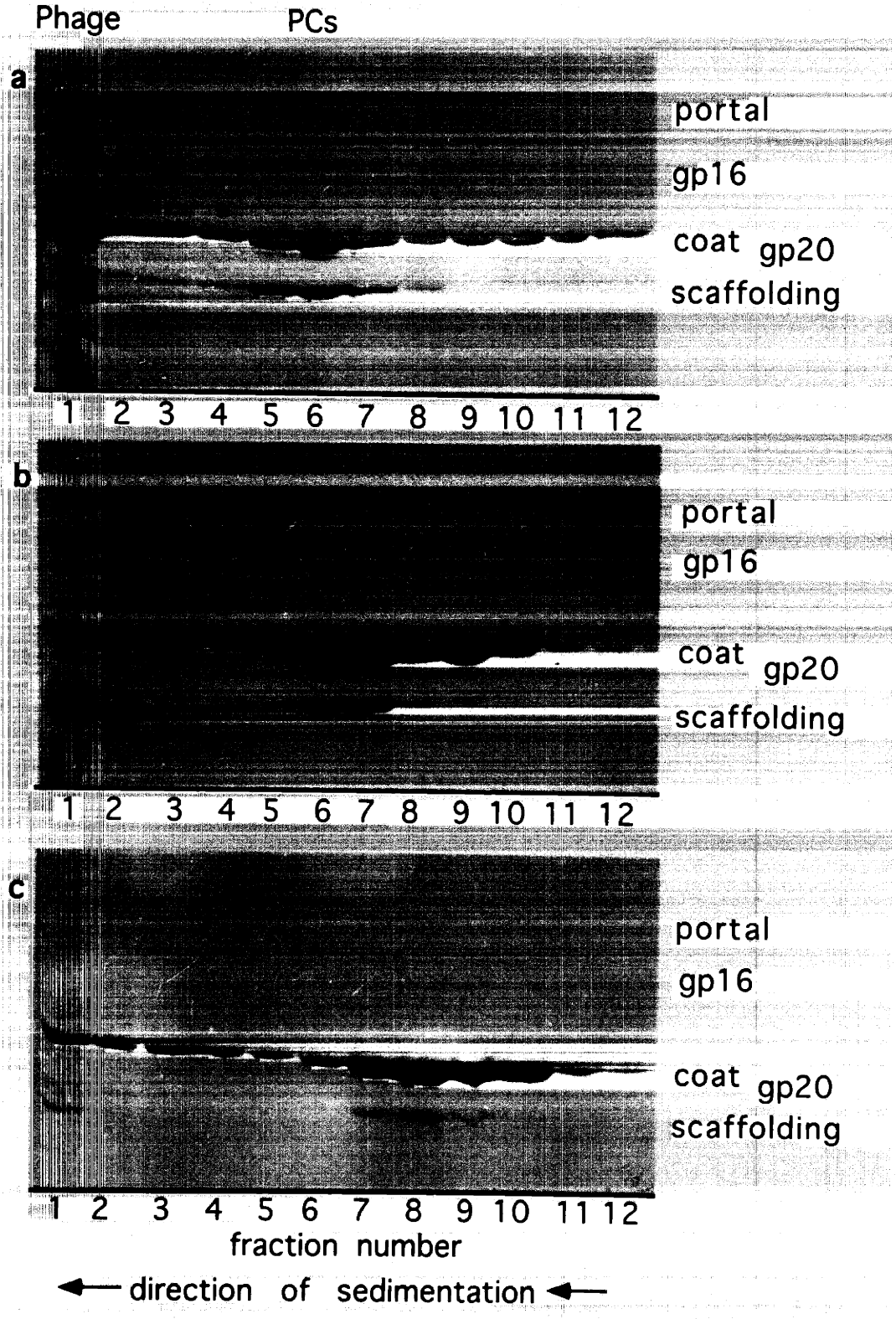
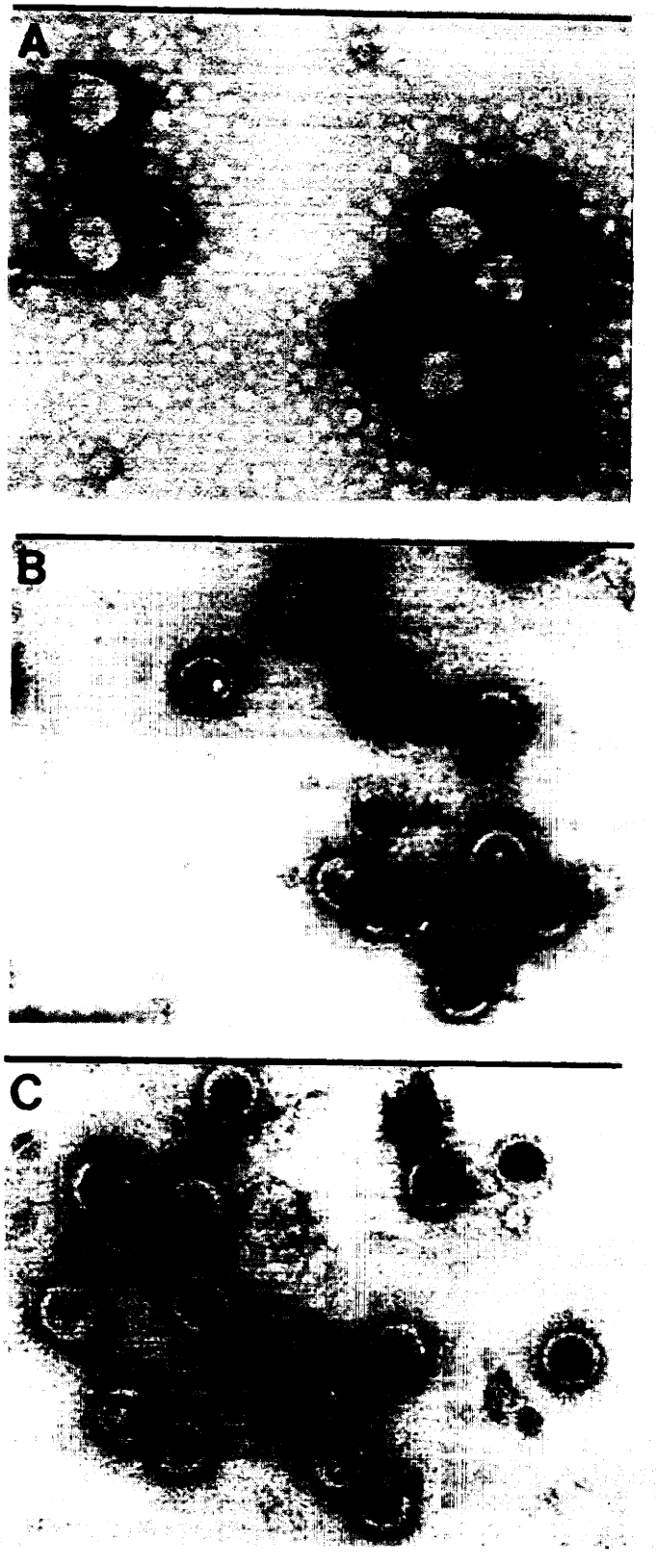

Phage
PCs
a
portal
gp16
coat gp20
scaffolding
1 2 3 4 5 6 7 8 9 10 11 12
b
portal
gp16
coat gp20
scaffolding
1 2 3 4 5 6 7 8 9 10 11 12
c
portal
gp16
coat gp20
scaffolding
1 2 3 4 5 6 7 8 9 10 11 12
fraction number
direction of sedimentation

FIG. 3.6 Electron microscopy of structures produced by 8 cs mutants at 17°C. The lysates subjected to sucrose gradient centrifugation in Fig. 3.5 were observed by negative stain at a magnification of 60,000 times. (a) wild-type, (b) 8cs/ts Q149W, (c) 8cs Q149Y.


A
B
C

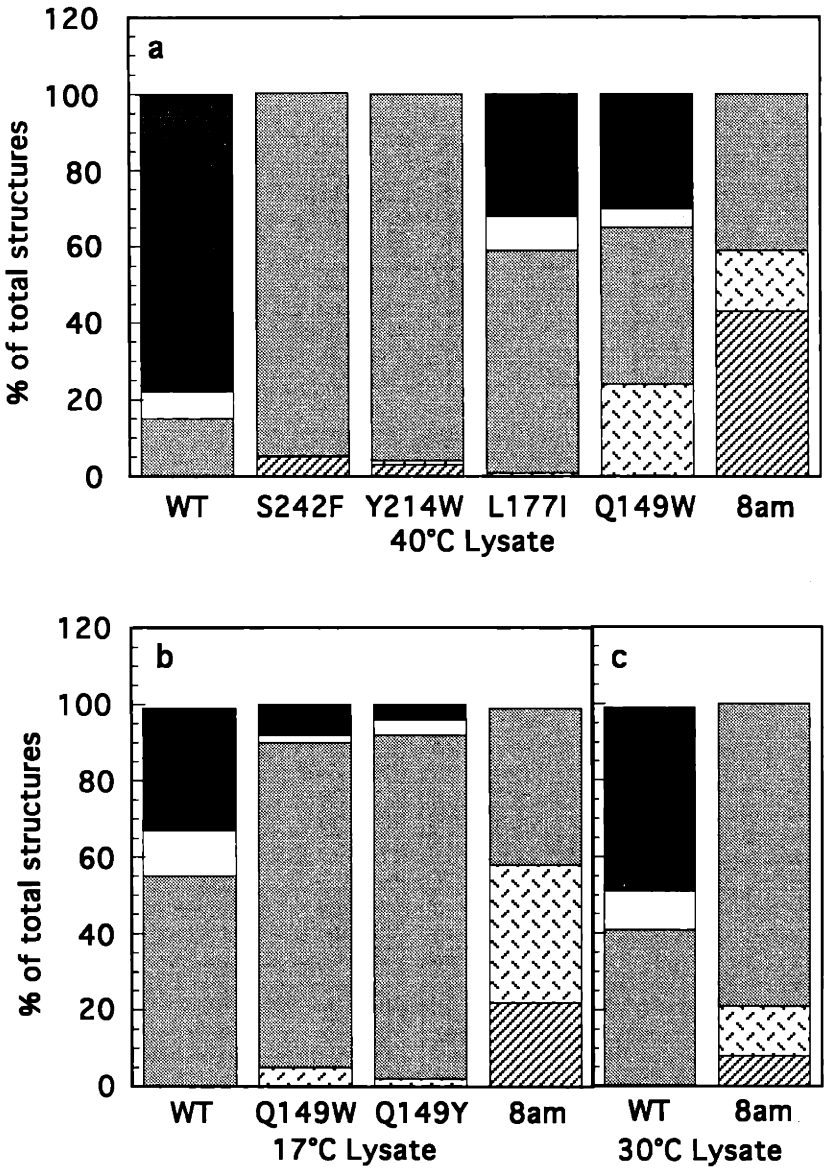


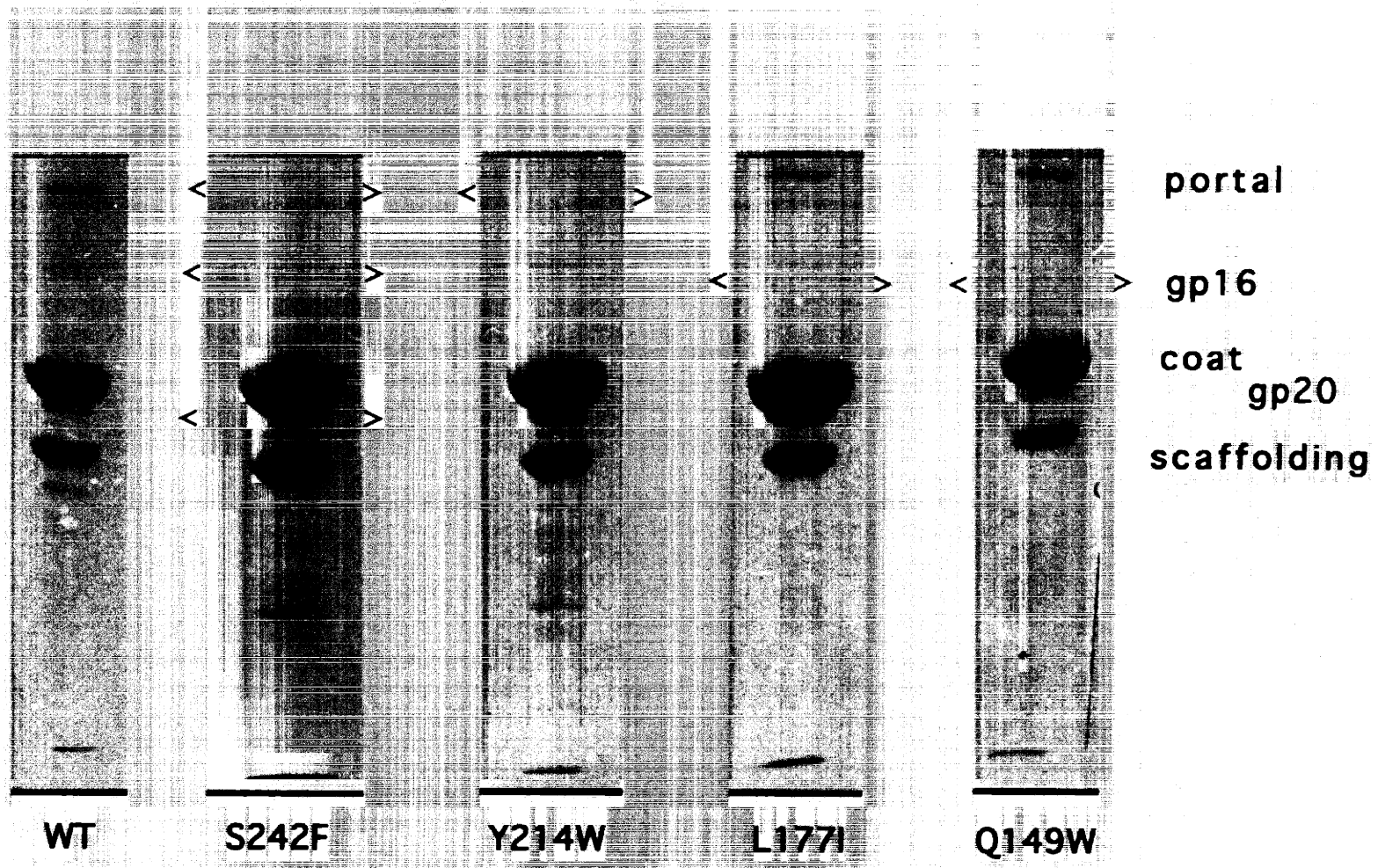

FIG. 3.7 Structures produced by scaffolding mutant strains at non-permissive temperature. The structures observed in electron micrographs of infected cell lysates were counted and classified according to the criteria described in Materials and Methods. (a) Lysates from cells infected at 40°C, (b) 17°C, (c) 30°C controls. Black = phage, white = empty heads, gray = procapsids (or empty procapsids), crosshatching = aberrant structures, stripes= small capsids.

**Phenotypes of mutants with DNA packaging blocked**

The inability of the S242F and Y214W procapsids to progress to mature phage is readily explained by the fact that these capsids do not include portals, thus precluding the possibility of DNA packaging. It was more difficult to explain why the L177I procapsids are inhibited in maturation, since the presence of gp16 is not required for either assembly or DNA packaging (Botstein et al, 1973; Bryant and King, 1984). It was also not clear whether the procapsids made by the cold-sensitive mutants had lost their scaffolding subsequent to abortive DNA packaging attempts, or whether the premature exit of scaffolding in some way hindered DNA packaging. To address these issues, all of the mutations were crossed with an amber mutation in gene 2, whose product is required for DNA packaging; thus, these strains will all accumulate procapsids. As expected, all strains produced procapsids of normal composition at 30°C.

At 40°C, the ts mutants all produced procapsids, whose protein compositions are shown in Fig. 3.8. With DNA packaging blocked, the phenotypes of S242F and Y214W at 40°C were the same as previously observed, as expected, since these capsids cannot package DNA in the wild-type background. The double mutant L177I/2am produced only procapsids, which lacked gp16. The lysate of the double mutant Q149W/2am at 40°C contained a higher proportion of morphologically normal procapsids than observed in the absence of the DNA packaging block. These particles were presumably the precursors to the non-infectious phage particles observed in the wild-type background. It was apparent from SDS-PAGE that these particles did not contain gp16, as with L177I, revealing why they cannot mature into infectious phage.

FIG. 3.8 Protein composition of procapsids produced by 8ts mutants at 40°C with DNA packaging blocked. Lysates of cells infected with 8ts/2am double mutant strains were prepared and centrifuged through 5-20% sucrose gradients. The gradients were fractionated and the location of the capsid peaks determined by SDS-PAGE. Only the peak fractions are shown here. The positions of proteins absent from mutant procapsids are indicated by brackets.

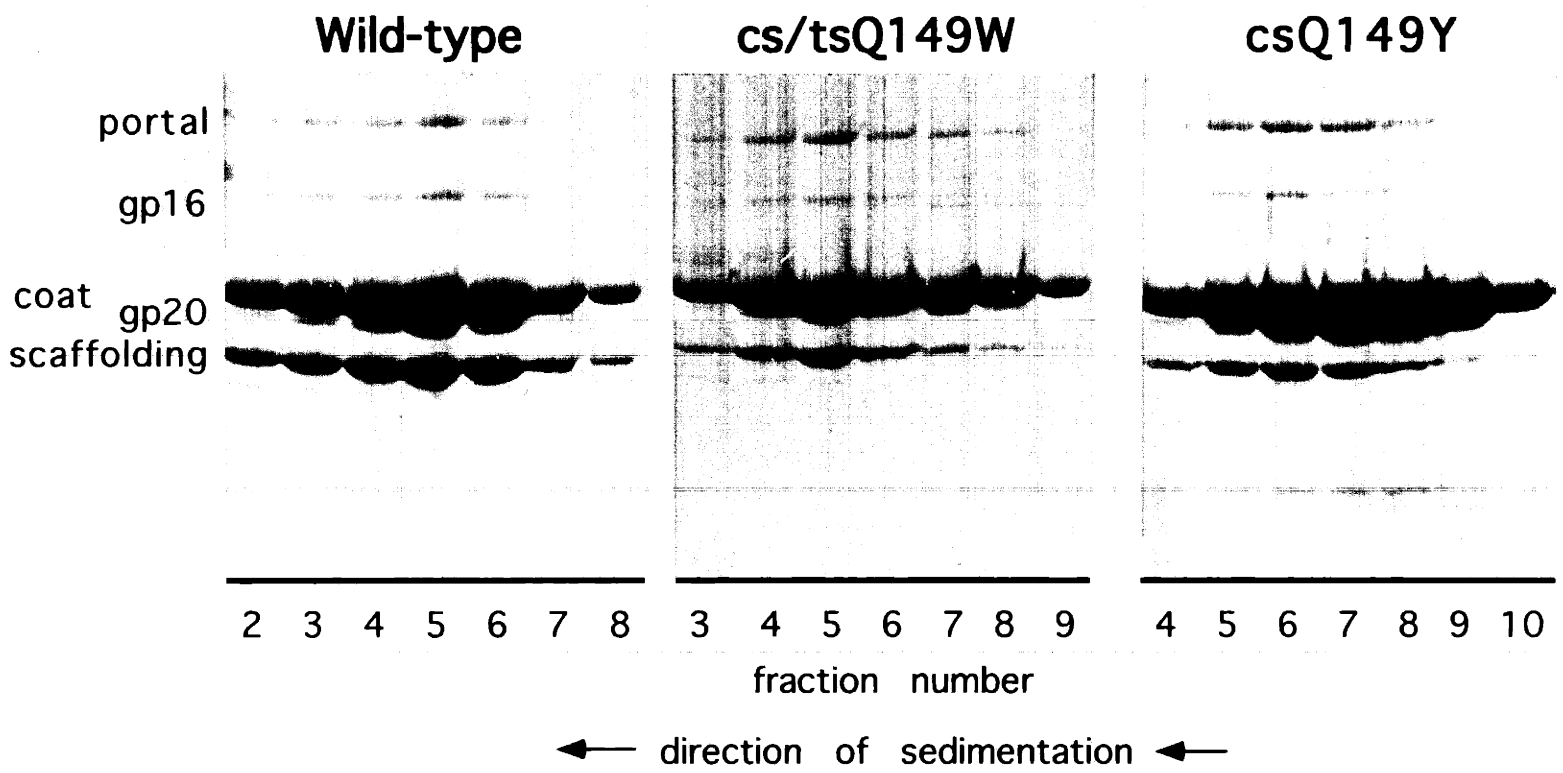

portal
gp16
coat
gp20
scaffolding
WT
S242F
Y214W
L177I
Q149W

Even with DNA packaging blocked, the cold-sensitive mutants still accumulated many empty capsids at low temperature, demonstrating that the scaffolding had not been released by initiation of DNA packaging. The sucrose gradients shown in Fig. 3.9 indicated a mixed population of particles, some of which contained more scaffolding, suggesting that all procapsids may have originally contained scaffolding, but that it had prematurely "leaked" from many. These capsids also contained decreased amounts of gp16 and gp20, but as these proteins are easily released by conditions which extract scaffolding it is more likely that they have leaked out of the assembled capsids than that the capsids were assembled without them. It is not clear why the loss of scaffolding protein should prevent DNA packaging, as the scaffolding must normally be released in any event. This phenotype is the same as that observed for the two cs mutants in the N-terminal region of the coat protein (Gordon, Lee, Reiner and King; Greene and King, unpublished results).

**Scaffolding release**

Since I was unable to explain the inabilty of some of the mutants to package DNA, I decided to test *in vitro* their ability to undergo the processes normally coupled with DNA packaging, such as the expansion of the procapsid to the mature capsid form and scaffolding release. Guanidine hydrochloride at only 0.5 M is sufficient to extract all the scaffolding from wild-type procapsids (Prevelige et al., 1988; Chapter 2). Procapsids were purified from cells infected with the five double mutant strains blocked in DNA packaging. The infections were carried out at the permissive temperature, so that the procapsids synthesized by the five mutant strains all had equivalent protein compositions. Scaffolding was extracted from these procapsids by incubation in a range of GuHCl concentrations at room

FIG. 3.9 Protein composition of procapsids produced by 8cs mutants at 17°C with DNA packaging blocked. Lysates of cells infected with 8cs/2am double mutant strains were prepared and centrifuged through 5-20% sucrose gradients. 18 fractions were collected from each gradient and their protein composition dtermined by SDS-PAGE. Fractions including the procapsid peaks from each gradient are shown.


Wild-type
cs/tsQ149W
csQ149Y
portal
gp16
coat
gp20
scaffolding
2 3 4 5 6 7 8
3 4 5 6 7 8 9
4 5 6 7 8 9 10
fraction number
direction of sedimentation

temperature. It was not possible to perform this experiment with Q149Y procapsids, as much of the scaffolding was already lost by the end of the purification process, which involved many steps at 4°C.

As shown in Fig. 3.10, the extraction of scaffolding from procapsids made by S242F and Y214W at permissive temperature had a similar profile to that observed for wild-type. Extraction of L177I procapsids was markedly different; even 1.0 M GuHCl was insufficient to release more than about half of the scaffolding. The difference for Q149W was less dramatic, but this mutant scaffolding was still clearly more difficult to release than wild-type, an unexpected result given its apparent leakiness at low temperature. The extraction process for the Q149W mutant procapsids also appeared to occur in two steps, with an intervening plateau from 0.4 to 0.6 M GuHCl. Perhaps these steps represent the release of the two classes of scaffolding molecules previously suggested to exist at alternate binding sites within the wild-type procapsid (Chapter 2). In the case of L177I, it may be that one class of molecules is completely unable to be extracted.

### *In vitro* expansion

Procapsid expansion *in vivo* is coupled to packaging of the DNA. The expansion can be triggered *in vitro* in the absence of DNA by a variety of treatments, including heat. This *in vitro* expansion of procapsids to the mature form is an exothermic reaction when followed by calorimetry (Galisteo and King, 1993). After heating at 65°C for 20 minutes, about half of the wild-type procapsids were found to undergo expansion, as observed by migration through an agarose gel (Fig. 3.11a). The degree of expansion was similar regardless of the temperature at which the wild-type procapsids had been produced.

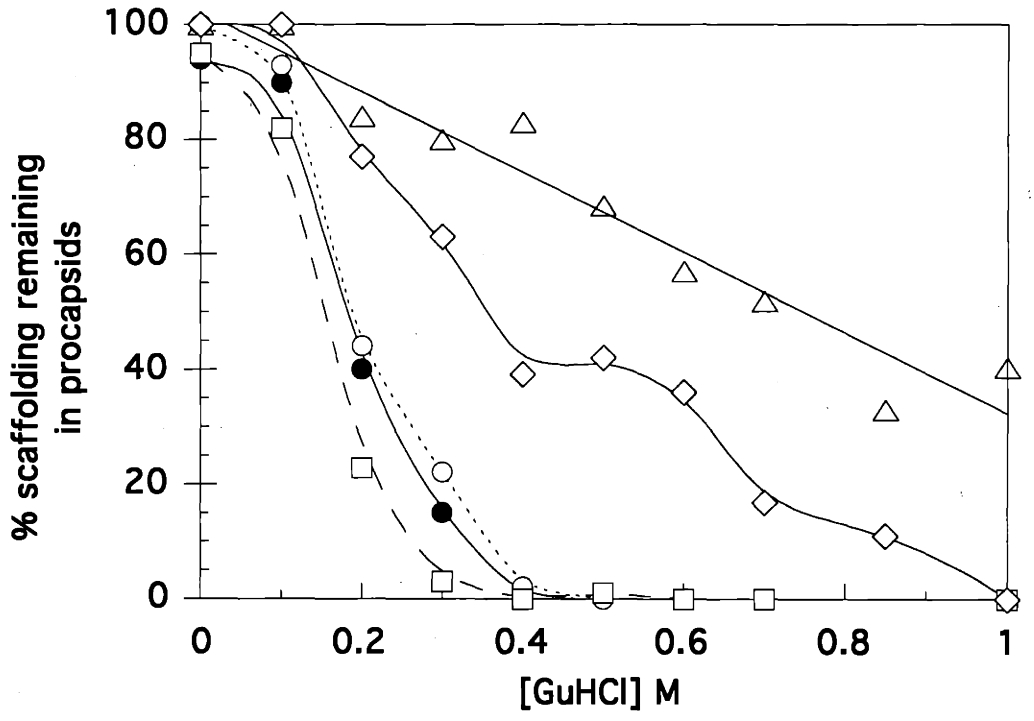



FIG. 3.10. GuHCl-induced extraction of mutant scaffolding proteins from procapsids. Procapsids purified from cells infected with 2am or 8ts/2am double mutant strains at the permissive temperature were incubated at 1.5 mg/ml in varied concentrations of GuHCl. Extracted scaffolding was separated from procapsids by sucrose gradient sedimentation. The protein composition of the gradient fractions was analyzed by SDS-PAGE. The amount of scaffolding remaining within the capsids and that sedimenting as monomers were quantified by densitometry of the Coomassie stained gels. Filled circles = wild-type, open circles = S242F, squares = Y214W, triangles = L177I, diamonds = Q149W.

The mutant procapsids fell into three classes with respect to the expansion reaction: L177I, which failed to expand; Q149Y/W which expanded more readily than wild type, and S242F and Y214W which displayed conditional expansion.

The procapsids of L177I produced at both temperatures did not expand, indicating that some property of this mutant scaffolding had trapped the coat lattice in the unexpanded state.

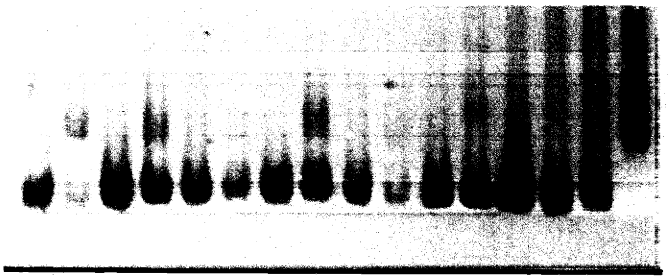
The procapsids made by mutants Q149W and Q149Y not only expanded, but expanded more readily than wild-type procapsids, as no unexpanded capsids were left after 20 minutes of heating (Fig. 3.11b). This property did not depend on the temperature at which the mutant procapsids were made. The unheated particles migrated to the same position as procapsids, demonstrating that the empty particles produced at low temperature were not already expanded. Procapsids made with the two N-terminal coat cs mutants, including the one suppressed by L177I, also expanded more completely than wild-type (not shown).

The Q149Y/W procapsids which expanded more readily than wild-type also leaked scaffolding at low temperature. In contrast, the capsids produced by L177I contained more tightly bound scaffolding and did not expand. I suspected that release of scaffolding might be a prerequisite for procapsid expansion, so that capsids from which scaffolding was more easily released would also expand more readily.

To test this idea I extracted the scaffolding from mutant procapsids made at the permissive temperature, to yield shells containing only coat protein. If the aberrant expansion properties were due to differences in the extraction of the scaffolding proteins by heat, then the empty shells should all demonstrate the same expansion characteristics. This was not the case; the

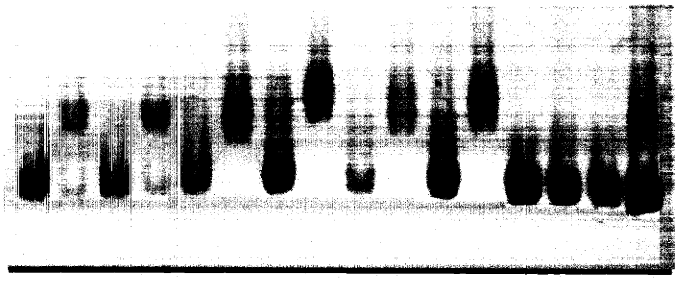
FIG. 3.11. *In vitro* expansion of wild-type and mutant procapsids and shells. Procapsid and shell samples at 1 mg/ml were either untreated (u) or heated (h) for 20 minutes at 65°C. Samples were mixed with Serwer sample buffer and the expanded and unexpanded capsids separated by electrophoresis though 1.8% agarose gels. (a) and (b) Lysates of cells infected with 8ts/2am mutants at the designated temperature, (c) purified shells with scaffolding extracted.

a



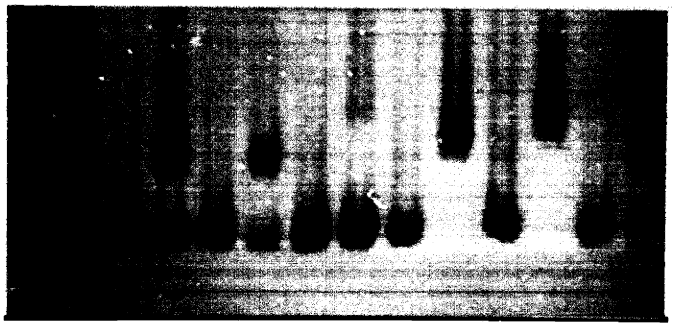
u h u h u h u h u h u h u h u h u h
40 30 40 30 40 30 40 40
wt S242F Y214W L177I Q149W

b



u h u h u h u h u h u h u h u h u h
30 17 30 17 30 17
wt Q149W Q149Y 1- PCs
8-shells

c



u h u h u h u h u h u h u h u h
wt SF YW LI QW QY wt PCs
Empty Shells

shells derived from L177I procapsids did not expand, while those from Q149W and Q149Y still expanded more completely than wild-type shells (Fig. 3.11c). It was not possible to fully extract all the scaffolding from the L177I procapsids even after multiple extractions with GuHCl, so the failure of these shells to expand might be due to residual tightly bound scaffolding. Nonetheless the Q149Y/W results suggest that the mutant scaffoldings might alter the coat lattice, or the packing of the portal ring into the coat lattice.

The procapsids produced by the mutant S242F at 30°C underwent expansion to the same degree as wild-type. However, procapsids produced by S242F formed at 40°C failed to expand in the gel assay. The same permissive and restrictive phenotypes were observed for the mutant Y214W. Since the procapsids produced by both these mutants lack portals at the high temperature, it was possible that the portal was needed for the expansion reaction.

To test this possibility, procapsids were isolated from cells infected with phage carrying an amber mutation in gene 1, the portal protein gene. The N-terminal portal fragment produced in these cells is not incorporated into the procapsids assembled in these cells. These procapsids composed of wild type scaffolding protein but lacking portal rings failed to expand *in vitro*, suggesting that the portal was required for the initiation or propagation of expansion.

I also examined the behavior of the normally sized capsids lacking the portal and other minor proteins that are assembled in cells infected with phage bearing an amber mutation in the scaffolding gene. These are the "8$^-$ shells" described in Chapter 2. Although not containing portals, these shells expanded to the same extent as wild-type procapsids. This result suggested that the expansion reaction does not require a portal structure.

This paradox may be resolved by noting that the 8⁻ shells differ from the portalless procapsids in lacking scaffolding. The inability of L177I procapsids to expand, and the failure to observe expanded capsids containing scaffolding in any system, imply that scaffolding release may be a prerequisite for capsid expansion. The presence of the portal might be required to initiate not expansion itself, but scaffolding release. This would explain previous observations that higher GuHCl concentrations are required to extract wild-type scaffolding from portalless procapsids, and that this extraction process is less cooperative than observed with procapsids containing portals (B. Greene, unpublished results). In this case, while lack of a portal would block expansion by impairing scaffolding release, the absence of both portal and scaffolding would not affect expansion.

**Recessive/dominant properties and intragenic complementation**

As the four ts mutants have distinct phenotypes, I was curious as to whether they would exhibit intragenic complementation. Perhaps a mutant defective in portal insertion would complement one defective in scaffolding release. Though intragenic complementation has been best defined for discrete multimeric enzymes (Garen and Garen, 1963; Coddington and Fincham, 1965; Zabin and Villarejo, 1975) intragenic complementation has been observed for cs mutants in the coat protein (Gordon and King, 1994).

Mixed infections were carried out under restrictive conditions to test for intragenic complementation. Mixed infections with wild type were also performed to determine whether the scaffolding alleles were recessive or dominant. All of the mutants were recessive to wild type (Table 3.3). None of the mixed infections between scaffolding mutant alleles showed evidence of complementation. This may be because all the mutants are defective in

TABLE 3.3

Complementation Tests with Scaffolding TS Mutants

| Infecting Phage | Burst at 30°C | Burst at 40°C |
|---|---|---|
| wild-type | 265 | 525 |
| scaffolding amber | <2 | <2 |
| scaffolding amber + wt | 65 | 288 |
| S242F | 248 | <2 |
| S242F + wt | 220 | 300 |
| Y214W | 333 | <2 |
| Y214W + wt | 235 | 418 |
| L177I | 470 | 3 |
| L177I + wt | 393 | 183 |
| Q149W | 248 | <2 |
| Q149W + wt | 430 | 250 |
| S242F + Y214W | 363 | <2 |
| S242F + L177I | 763 | 5 |
| S242F + Q149W | 370 | 2 |
| Y214W + L177I | 603 | <2 |
| Y214W + Q149W | 325 | 2 |
| L177I + Q149W | 305 | <2 |

incorporation of one or more minor proteins, which probably all must be included at the same time during assembly.

**Overexpression of GroE**

Temperature-sensitive mutants in the coat protein can be rescued at high temperature by overexpression of the chaperone GroE (Gordon et al, 1994). The GroE appears to prevent aggregation of the mutant coat proteins. There is no indication that the scaffolding protein requires the assistance of GroE to fold. However, GroE is required for the initial steps of lambda procapsid assembly, the formation of the dodecameric portal complex, a process which also involves the lambda scaffolding protein (Murialdo and Becker, 1978b; Murialdo, 1979; Georgopoulos et al., 1983). Since two of the P22 scaffolding mutants affect the insertion of the portal, I thought it possible that supplying extra GroE might help to overcome this defect. All four scaffolding ts mutants were plated at a range of temperatures onto cells carrying the either the *E. coli* GroE genes on a plasmid or a control plasmid (Table 3.4). The temperature-sensitive coat mutant used as a control showed an increase in phage production of 6 orders of magnitude when plated on the GroE overexpressing cells at 37°C. By comparision none of the ts scaffolding mutants showed significant rescue at any temperature. P22 can grow on cells bearing missense mutations in GroE (C. Teschke, unpublished results), which together with these results suggests that GroE may not be involved in P22 portal assembly.

TABLE 3.4

Effect of GroE overexpression on scaffolding temperature-sensitive mutants

| Mutant | GroE | Efficiency of Plating | | | | |
|---|---|---|---|---|---|---|
| | | 30°C | 35°C | 37°C | 39°C | 41°C |
| 5tsG232D | - | 1.0 | | $3x10^{-6}$ | $6x10^{-7}$ | |
| | + | 1.0 | | 0.7 | $6x10^{-7}$ | |
| 8tsS242F | - | 1.0 | | 0.4 | $2x10^{-4}$ | |
| | + | 1.0 | | 0.6 | $2x10^{-4}$ | |
| 8tsY214W | - | 1.0 | | 0.4 | $1x10^{-4}$ | $7x10^{-7}$ |
| | + | 1.0 | | 0.7 | $2x10^{-4}$ | $9x10^{-7}$ |
| 8tsL177I | - | 1.0 | | 0.8 | $2x10^{-4}$ | $8x10^{-7}$ |
| | + | 1.0 | | 0.9 | $2x10^{-4}$ | $4x10^{-7}$ |
| 8ts/csQ149W | - | 1.0 | 0.4 | $2x10^{-4}$ | $1x10^{-4}$ | $2x10^{-7}$ |
| | + | 0.9 | 0.5 | $3x10^{-4}$ | $1x10^{-4}$ | $3x10^{-7}$ |

# DISCUSSION

## Isolation of new missense mutants in the scaffolding gene

New mutants were successfully isolated by screening revertants of existing amber and missense mutations as described by Jarvik and Botstein (1975). Another strategy would have been extensive mutagenesis of a local region, as has recently been done to isolate mutations affecting four different functions of yeast calmodulin (Ohya and Botstein, 1994). However, these protocols are typically guided by the availability of the three-dimensional structure and prior knowledge of the functional domains, information that was not available for the P22 scaffolding protein. The failure to obtain mutants in the scaffolding gene by random mutagenesis suggested that many sites might be either highly tolerant or intolerant to substitution. Any targeted mutagenesis of the 303 amino acid scaffolding protein would thus probably have a very low likelihood of generating useful substitutions. The strategies used here allowed targeting of the scaffolding gene, unlike a general mutant hunt, while the screens used to identify mutant phage guaranteed that any mutants obtained would have a phenotype.

Temperature sensitive mutations in both the tailspike and coat protein cause the folding pathway to shift from productive folding to aggregation (Haase-Pettingell and King, 1988; Gordon and King, 1993). At high temperature, the ts coat mutants do not form any regular structures, but aggregate into inclusion bodies (Gordon and King, 1993). This does not appear to be the case with the scaffolding ts mutants, suggesting that their folding is less thermolabile. Another possible phenotype would have been scaffolding proteins completely unable to associate with coat protein. Yet all these mutants have clearly different phenotypes from the scaffolding amber strain,

and all are found within coat particles. It appears that the mutants do selectively disrupt only particular functions of the protein.

**Incorporation of minor proteins**

A critical role for scaffolding protein in the incorporation of the portal and the pilot proteins had previously been inferred from the absence of minor proteins from the aberrant coat structures formed in scaffolding amber infections (Earnshaw and King, 1978), as well as from the phenotype of S242F (Bazinet and King, 1988). This evidence suggested that the scaffolding was a key component of an initiation complex that recruited the portal and pilot proteins. The phenotypes of the new mutants, however, suggest that the actual process may be more complex. Since Y214W includes gp16 and gp20, but not the portal, while L177I and Q149W include the portal and gp20 but not gp16, the mechanisms by which all these proteins are included in the capsid may not be the same.

The role of gp16 in procapsid assembly has been studied by Prevelige and Thomas (1991), who found that its presence accelerated the rate of initiation of *in vitro* procapsid assembly. While evidence from cosedimentation studies suggested that gp16 could interact with the coat protein, no indication of any interaction between gp16 and scaffolding was observed. Perhaps the exclusion of gp16 from L177I and Q149W procapsids is a secondary effect, rather than an indication of a defect in direct scaffolding/gp16 bonding.

It seems reasonable to suggest that the mutations S242F and Y214W, less than 30 amino acids apart in the sequence, affect a region involved in portal insertion, either by direct interaction with portal protein or by participation in an initiation complex that recruits the portal. Genetic

evidence from lambda (Murialdo and Becker, 1978b; Murialdo, 1979) and T4 (Laemmli et al., 1970) indicates that scaffolding is required for the first initiation steps of capsid assembly, in which the portal vertex is assembled.

Direct interaction between the portal and scaffolding proteins of phi29 was observed by gel shift assays (Guo et al., 1991). An amber mutation at position 172 of the 269 amino acid T4 scaffolding protein gp22 has a ts phenotype on a tyr-inserting host; a second site supressor of this mutation maps to gene 20, encoding the portal (Mesyanzhinov et al., 1990). The authors proposed that this mutant identified a region of the protein, modelled as an alpha helix, that interacted with the portal. This T4 mutant is in the C-terminal third of the protein, as are the P22 mutants defective in portal insertion. It seems likely that one essential role of phage scaffolding proteins is to incorporate the portal, and these mutations may identify the specific region in the P22 protein responsible for this activity, a region which may be conserved in other scaffolding proteins.

**Morphogenesis**

The aberrant shells generated by Q149W at high temperature are reminiscent of defects produced by temperature-sensitive mutants of the major T4 scaffolding protein, gp22 (Keller et al., 1988). These mutants generated procapsids of aberrant shapes that could be described as perturbations of the basic icosahedral symmetry (Kellenberger, 1990); some were isometric rather than prolate, or prolate but of insufficient length, while others were bi or triprolate. P22 procapsids are isometric, and so do not need as complicated a sizing mechanism as T4. The P22 aberrant capsids do not appear to have any recognizable pattern of defects; they may not even be closed shells, but related to spirals. Unlike spirals, however, they appear to

have included portals, showing that portal insertion and elongation are separable functions. In contrast to Phi29, in which the portal seems to determine the correct length of the prolate capsid (Guo et al., 1991), these mutant procapsids can insert the portal correctly, but still assemble wrongly, demonstrating that scaffolding plays the essential controlling role. However, these capsids are less defective than structures produced in scaffolding amber infections, perhaps indicating some role for initiation in controlling assembly.

I think it is more likely that the presence of the long scaffolding molecules within the growing capsid provides a steric constraint that prevents the coat protein from forming the more tightly wound spirals found in the scaffolding amber lysates. In addition, the elongated scaffolding molecules might be making longer range contacts that drag the edges of the growing shell into forming a closed, although oddly shaped capsid. Computer models of P22 procapsid assembly demonstrate that spirals can result from the insertion of a single hexamer in place of a pentamer (Berger et al., 1994). By allowing the coat protein subunits to look futher away for nearby subunits (as the addition of scaffolding molecules might allow) the structures could be made to close rather than spiral (B. Berger, personal communication).

To my surprise, a large majority of coat structures assembled in cells infected with a scaffolding amber mutant at 30°C were normally sized capsids. At both 40°C and 17°C the proportion of normal capsids is significantly lessened, with the difference at 40°C being made up not by spirals, but by small capsids. The proportions of structures observed at 40°C are consistent with those reported by Earnshaw (1977), from cells infected at 37°C. The intrinsic curvature of the coat protein may be altered at high temperature, or there may be kinetic effects involved. This phenomenon may be responsible

for the presence of small capsids, normally a rarity in preparations of wild-type procapsids, in cells infected with mutants S242F and Y214W at 40°C (Figs. 3.4 and 3.7).

**Scaffolding release**

The mutations at sites 177 and 149 affect the process of scaffolding extraction *in vitro*. If the scaffolding is also more difficult to release *in vivo*, that could account for the inefficiency of the procapsids produced by these mutants in packaging DNA.

What is the mechanism of this defect? The sites of these mutations may be part of a domain involved in binding to the coat protein. By altering residues that contact the coat protein, the mutations might make the binding surface "stickier" at high temperature. Other results (discussed in Chapter 4) indicate that the extreme C-terminus is an essential binding region, but this area might be close to the C-terminus in the folded protein, or may make a secondary interaction. However, scaffolding constructs beginning at residue 180 are able to assemble coat subunits into procapsids (S. Casjens, personal communication), which makes it difficult to see how the region from 149-177 could be an essential coat-binding domain.

This region might instead mediate a scaffolding/scaffolding interaction. Since the scaffolding subunits probably must be monomeric in order to fit through the exit channels, substitutions that cause the mutant scaffolding proteins to associate more tightly would prevent them from exiting the procapsid.

Another possibility is that the release of scaffolding is not just a cessation of binding, but an active process in its own right, involving a new functional region. In this model, the docking of the DNA packaging complex

to the portal would transmit a signal to the nearby scaffolding molecules. The receipt of this signal would induce a conformational change that would propagate throughout the capsid and cause the scaffolding subunits to cease binding. Some active switch would seem to be required, since the scaffolding in procapsids is normally stable and does not spontaneously diffuse out. The L177I mutant may be defective in this "release switch" so that the switch is not well triggered, or the signal not propagated. The scaffolding may diffuse out of the cs mutants because their switch is too easily triggered.

As can be seen in Fig. 3.7, the ratio of mature phage to procapsids in wild-type lysates is significantly higher at 40°C than 30°C or 17°C, suggesting that the rate of DNA packaging increases with temperature. Similar results have been observed by J. Jarvik (1975). The DNA packaging enzymes may function more rapidly at higher temperature. On the other hand, the scaffolding protein can be partially extracted by incubation at temperatures in the range of 40 – 50°C (Galisteo and King, 1993). It may be that the release of scaffolding is an intrinsically slow step in phage maturation.

**Capsid expansion**

It is still unclear why the cold-sensitive mutants do not package DNA, since the release of scaffolding protein is a normal part of the wild-type maturation pathway. Models where scaffolding release is energetically coupled to DNA packaging have been proposed, but are not overly convincing, as in related phages such as lambda DNA can be packaged into capsids empty of scaffolding (Earnshaw and Casjens, 1980). The cs mutant capsids are clearly able to continue on the maturation pathway by undergoing expansion; in fact, they expand more readily than wild-type. This effect is not due to the loss of scaffolding from the capsids, as wild-type shells from which

scaffolding has been extracted *in vitro* do not display this altered expansion. That two coat protein mutants share this phenotype supports the idea that it is due to differences in the structures of the capsids. The differences must be subtle, since the capsids tested were made at the permissive temperature, and presumably would be able to mature into phage *in vivo*. Perhaps these capsids are a small step further along the expansion pathway than wild-type, and so have prematurely passed the stage at which they are competent for DNA packaging.

**An expanded pathway for P22 assembly**

A more detailed model of the P22 assembly pathway is shown in Fig. 3.12, with the likely points of the mutant defects indicated. In this picture, the first assembly step is the formation of an intitiation complex containing scaffolding and the minor proteins, and perhaps the first few coat subunits. Extension of the capsid depends upon different scaffolding regions, that control the recruitment of new coat subunits and the manipulation of their curvature to form a closed shell. This process probably involves two different scaffolding sites, one to bind the coat protein, and another to bind the scaffolding subunits to each other, thus pulling the coat subunits into a closed capsid. Finally, scaffolding release is a distinct process, in which the docking of the DNA packaging complex at the portal triggers a "release switch" in nearby scaffolding subunits. This conformational change is propagated thoughout the scaffolding core so that all the scaffolding molecules cease to bind the coat and exit through the channels. Freed of scaffolding, the coat lattice is able to expand and close the channels. The capsid may then proceed through one or more intermediate states before reaching the final expanded conformation.

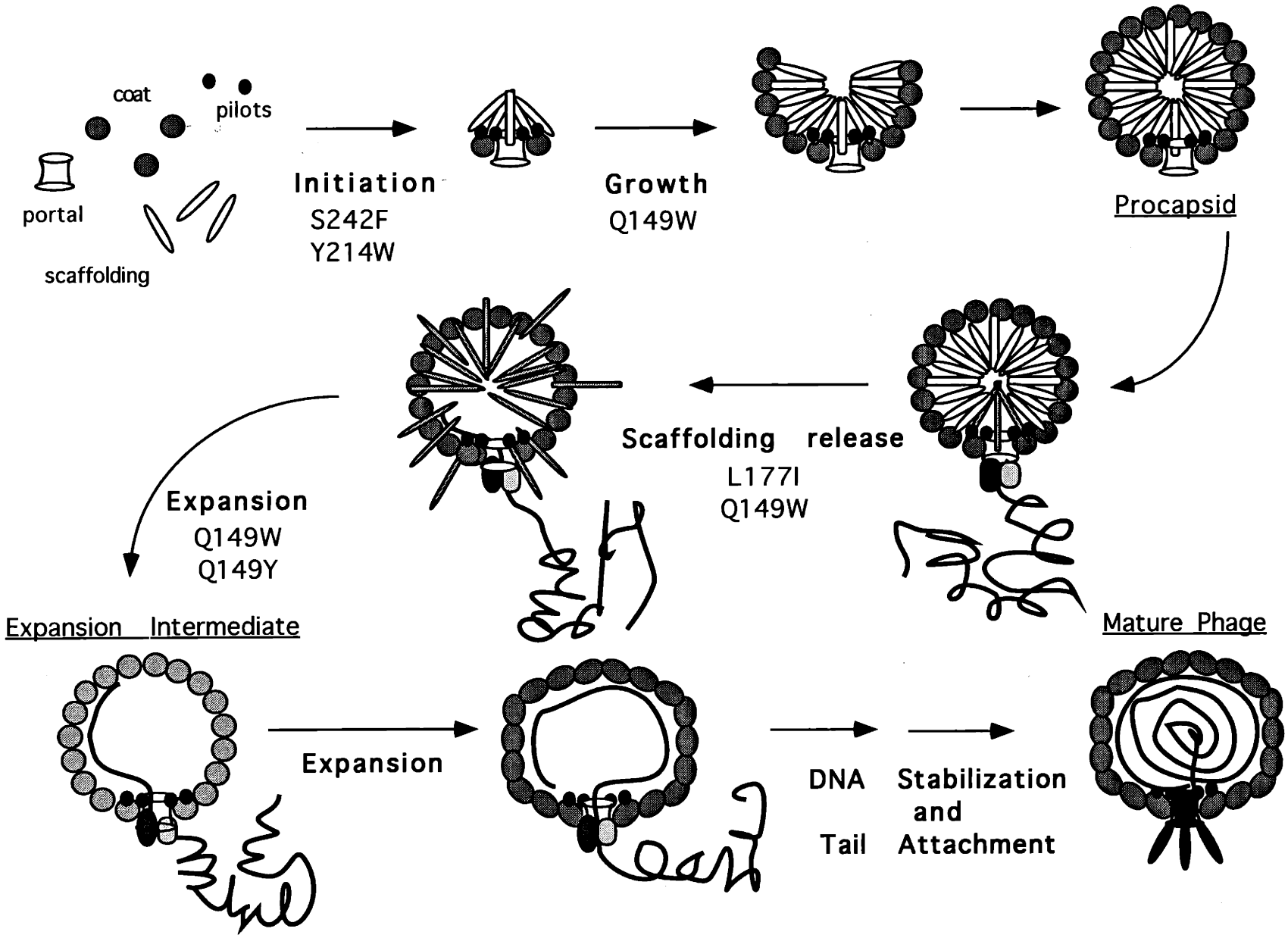



FIG. 3.12 An expanded assembly pathway for bacteriophage P22

Viruses are often perceived from the static perspective of a high-resolution mature stucture. In this model, I hope to stress the dynamic nature of the assembly process. It is generally recognized that structural proteins must undergo conformational changes during assembly. The results of this chapter indicate that associated assembly proteins are not passive support beams or templates, but also undergo active transformations.


## ACKNOWLEDGMENTS

Two undergraduate students contributed to the work discussed in this chapter. Morgan Kelly, as a summer student in the Research Science Institute program, performed some of the screens for pseudorevertants of scaffolding amber mutations and isolated the new mutant Y214W. Raka Mustaphi, a UROP student from MIT, carried out several experiments designed to study the binding of mutant scaffolding proteins to the coat lattice. The GuHCl extraction data shown in Fig. 3.10 were a part of her work.

# CHAPTER 4

# FUNCTIONAL DOMAINS OF BACTERIOPHAGE P22 SCAFFOLDING PROTEIN

## INTRODUCTION

Scaffolding proteins are essential for the formation of several classes of virus, including dsDNA bacteriophage (Casjens and Hendrix, 1988), herpesviruses (Rixon, 1993), and adenoviruses (Horwitz, 1991). These proteins are required for assembly of the viral capsid, but not found in the mature virus after DNA packaging. In the absence of scaffolding proteins, the viral coat protein polymerizes into aberrant structures that cannot package DNA (Casjens and Hendrix, 1988; Matusick-Kumar et al., 1995; Thomsen et al., 1995).

That scaffolding proteins have functions in addition to morphogenesis is growing increasingly evident. The process by which the dsDNA phages insure the incoporation of a DNA packaging portal at a unique vertex (reviewed in Bazinet and King, 1985) requires scaffolding protein. Interactions between scaffolding and portal proteins have been inferred genetically (Murialdo and Becker, 1978a; Laemmli, 1970; Bazinet and King, 1988) or observed directly, in the case of phi29 (Guo et al., 1991). The herpes simplex virus scaffolding protein is required for nuclear localization of the viral coat protein (Nicholson et al., 1994; Matusick-Kumar et al., 1994). Mutations within a specific region of the P22 scaffolding affect the ability of the scaffolding to be released from the procapsid (Chapter 3), suggesting that release, or sensing of DNA entry, may be another scaffolding function.

These results demonstrate that scaffolding proteins are not simply support beams, but complex, multifunctional proteins. Unfortunately, little is known about scaffolding protein structures. Although many virus structures have been revealed by x-ray crystallography (Rossmann and Johnson, 1989) or cryo-electron microscopy and image reconstruction (Stewart and Burnett, 1994) these structures were mature viruses that did not contain scaffolding. No scaffolding protein from any virus has yet been crystallized.

As mentioned in the Introduction, analysis of the sequences of several phage scaffolding proteins suggested that they all tend to be predominatly alpha-helical in structure (Eppler et al., 1991). This prediction has been confirmed for the P22 scaffolding protein by Raman spectroscopy (Thomas et al., 1982) and circular dichroism (Teschke et al., 1993). Analytical ultracentrifugation or gel filtration have revealed that the scaffolding proteins of T4, lambda, and P22 are all highly elongated molecules (van Driel, 1980; Ziegelhoffer et al., 1992; Fuller and King, 1982). Scaffolding proteins may resemble many actin-binding proteins (ABPs) in being long rods containing a series of modular functional units (Matsudaira, 1991; Vandekerckhove, 1992). As there are no leucine zipper or heptad repeats evident in the P22 scaffolding sequence (Fig. 4.1), the protein is probably not a coiled-coil.

In the case of bacteriophage P22, several functions of the scaffolding protein have been localized to specific regions of the molecule. The unassembled scaffolding protein regulates its own synthesis at the translational level (King et al., 1978; Wyckhoff et al.,1985) presumably by binding to its mRNA. The region involved in this function has been localized to the N-terminus, since amber fragments down to one quarter the length of the intact protein are capable of repression (Casjens et al., 1985). As

**Amino Acid Sequence of Bacteriophage P22 Scaffolding Protein**

| | |
|---|---|
| M E P T T E I Q A T E D L T L S G D H A A A S A D S L V V D | 30 |
| N A N D N A G Q E E G F E I V L K D D E T A P K Q D P A K N | 60 |
| A E F A R R R I E R K R Q R E L E Q Q M E A V K R G E L P E | 90 |
| S L R V N P D L P P Q P D I N A Y L S E E G L A K Y D Y D N | 120 |
| S R A L A A F N A A N T E W L M K A Q D A R S N A V A E Q G | 150 |
| R K T Q E F T Q Q S A Q Y V E A A R K H Y D A A E K L N I P | 180 |
| D Y Q E K E D A F M Q L V P P A V G A D I M R L F P E K S A | 210 |
| A L M Y H L G A N P E K A R Q L L A M D G Q S A L I E L T R | 240 |
| L S E R L T L K P R G K Q I S S A P P A D Q P I T G D V S A | 270 |
| A N K D A I R K Q M D A A A S K G D V E T Y R K L K A K L K | 300 |
| G I R | |

| | | |
|---|---|---|
| Charged residues: | 31.7% | (a) |
| Calculated pI: | 4.91 | (a) |
| S value: | 2.4 | (b) |
| axial ratio: | 9:1 | (b) |

secondary structure composition: (c)

30% α-helix, 10% β-sheet, 20% turn, 40% random coil

FIG. 4.1 Amino acid sequence and other characteristics of bacteriophage P22 scaffolding protein. The scaffolding sequence is as reported in Eppler et al., 1991. (a) Data from Eppler et al., 1991, (b) Fuller and King, 1982, (c) Teschke et al., 1993.

discussed in Chapter 3, two mutations in the C-terminal third of the scaffolding sequence are defective in insertion of the DNA packaging portal, while two other mutations located in the middle of the sequence affect scaffolding release.

In this chapter, I present experiments designed to explore the structural organization of the scaffolding protein. I was particularly interested in determining the scaffolding region involved in binding to the coat protein. The actin binding domains of many ABPs have been determined by assaying the binding ability of proteolytic fragments (Matsudaira, 1992). Since there are now two assays for coat binding – the *in vitro* assembly system (Prevelige et al., 1988) and reentry into intact shells (described in Chapter 2) – this approach seemed promising. Proteolytic fragments of scaffolding protein were assayed for their ability to bind to coat shells or to promote assembly of coat monomers into procapsids *in vitro*.

Various protein denaturation techniques have been utilized as probes of protein structure. Many multidomain proteins exhibit non-two state denaturation curves, reflecting the serial unfolding of separate parts of the protein (Privalov, 1982). The denaturation of the wild-type protein by both GuHCl and heat was monitored by two signals, fluorescence and circular dichroism, to look for evidence of non two-state behaviour that would indicate the presence of multiple folding domains.

Single-tryptophan mutants have been utilized to probe the folding and stability of individual protein regions in the three-domain colicin E1 channel peptide (Steer and Merrill, 1995). Fortuitiously, the wild-type scaffolding protein is already a single-tryptophan protein, while two scaffolding mutations add new tryptophans that might serve as probes of new regions.

Since the phenotypes of these mutants are known (Chapter 3), the functions of any new regions would also be identified.

Alterations to a protein's sequence can selectively perturb the stability of single domains of a multidomain molecule. For example, certain mutations in the N-terminal DNA-binding domain of lambda repressor lowered the melting temperature of that domain without affecting the C-terminal oligomerization domain (Pabo et al., 1983; Hecht et al., 1986). Deletions in the N-terminus of human carbonic anhydrase II destabilized only the N-terminal domain, while the more stable C-terminal domain was unaffected (Aronsson et al., 1995). Since the four temperature-sensitive mutants described in Chapter 3 are defective in discrete functions, I thought it possible that they might affect specific domains of the scaffolding protein. If the mutants altered protein stability, rather than surface interactions, these effects could produce selective domain destabilization. Accordingly, I purified and characterized the thermal denaturation of all four temperature-sensitive mutant proteins.

## MATERIALS AND METHODS

### Chemicals

Ultrapure guanidine hydrochloride (GuHCl) was purchased from Pierce. Proteases (V8, chymotrypsin-agarose, trypsin-agarose) and inhibitors were ordered from Sigma. Molecular weight standards were purchased from Bio-Rad. All other chemicals were reagent grade from common sources.

### Purificatation of coat monomers and intact shells

Wild-type procapsids were purified from cells infected with a phage mutant blocked in DNA packaging, and both scaffolding monomers and empty coat shells purifed as previously described in Chapter 2. Shells of coat protein were dissociated into monomers by incubation in 2.0 M GuHCl, and loaded onto a Biogel A 0.5 column to separate the monomers from any remaining intact shells, as described in Prevelige et al., 1988. The coat monomers were dialysed overnight at a concentration of approximately 1.0 mg/ml to remove the guanidine. The concentration of the coat monomers was determined by measuring absorbance at 280 nm, based on an extinction coefficient of $4.45 \times 10^4$ L $mol^{-1}$ $cm^{-1}$ (Teschke et al., 1993).

### Purification of wild-type and mutant scaffolding proteins

Phage strains carrying the 8ts mutations crossed into a 2am/13am background were used for the preparation of procapsids containing mutant scaffolding proteins. Infections with these strains were carried out at 30°C. Apart from this the mutant procapsids were prepared by the same protocol as for wild-type. Procapsids were obtained from mutant-infected cells in amounts comparable to preparations of wild-type procapsids. Mutant scaffolding proteins were purified from procapsids using the protocol

described for wild-type, although a higher concentration, 0.9 M instead of 0.5 M GuHCl, was used to extract scaffolding from L177I and Q149W procapsids. The yield of L177I mutant scaffolding protein was somewhat less than that of the others since it was not possible to remove all the L177I scaffolding from procapsids even after three extractions with GuHCl.

Wild-type and mutant scaffolding proteins were dialysed into phosphate buffer (20 mM $K_2HPO_4$, 25 mM NaCl, pH 7.6 with HCl) before use. The concentration was checked by absorbance at 280 nm, based an extinction coefficient of: $1.61 \times 10^4$ L $mol^{-1}$ $cm^{-1}$ for wild-type, L177I and S242F; $2.08 \times 10^4$ L $mol^{-1}$ $cm^{-1}$ for Y214W; and $2.21 \times 10^4$ L $mol^{-1}$ $cm^{-1}$ for Q149W. The extinction coefficients of the wild-type and mutant proteins were calculated using the method of Johnson (1988).

**GuHCl-induced denaturation**

Wild-type scaffolding protein was added to tubes containing varying concentrations of GuHCl in the phosphate/NaCl buffer, to give a final protein concentration of 100 μg/ml (0.3μM) and stored for at least 12 hours at 4°C. The signal from these samples remained constant up to 24 hours later, demonstrating that they had reached equilibrium. The same samples were measured by both fluorescence and CD.

Fluorescence measurements were performed using a Hitachi F4500 spectrofluorimeter interfaced with a personal computer. The excitation wavelength was set to 280 nm and the emission wavelength was 330 nm. The excitation and emission slit widths were set to 2.5 and 5.0 nm, and the PMT voltage was set to 700 V. The temperature of the cuvette was maintained at 10°C. Circular dichroism measurements were made using an Aviv Model 60DS spectropolarimeter with a computer-controlled Peltier thermostat.

Circular dichroism ellipticity at 222 nm was measured for samples in a 0.5 cm pathlength cell. The spectral bandwidth was 1.5 nm and the averaging time was 30 seconds.

The fraction folded was determined by the formula $(X_{obs}-X_u)/(X_n-X_u)$, where $X_n$ is the fluorescence or CD of the native protein, $X_{obs}$ is the observed value, and $X_u$ is the fully denatured value. $X_n$ and $X_u$ were determined by linear extrapolation of the folded and unfolded baselines into the region of the transition at each GuHCl concentration (Creighton, 1987). The slopes of the baselines were determined by linear least-squares analysis using the Kaleidagraph software (Abelbeck). The corrected data was fit to an equation for either a two-state transition or the sum of two or three two-state transitions. In order to limit the number of variables in these equations, separate folded and unfolded baselines for each of the two or three transitions were not determined, but assumed to be accounted for by the initial correction.

**Proteolysis**

Wild-type scaffolding protein was dialysed into 20 mM phosphate buffer, pH 7.6 with phosphoric acid. The scaffolding solution was diluted to a final concentration of 0.6 mg/ml (18 μM) in either phosphate buffer or phosphate buffer with GuHCl, and incubated at 10°C for 2 hours to allow unfolding to occur. 2.5 μg of protease V8 was added to give a 1:10 ratio of protease to scaffolding. The samples were mixed on a Nutator platform rocker for the designated amounts of time. Proteolysis was halted by addition of trichloroacetic acid (TCA) to 10%. The precipitated samples were pelleted by a 4 minute spin in an Eppendorf microfuge, and the pellets washed with

acetone to remove the acid. Samples were analysed by SDS-PAGE on a 10% acrylamide gel.

**Assembly and binding of proteolytic fragments**

Wild-type scaffolding protein was diluted to 1 mg/ml (3 μM) in 200 μl of phosphate buffer. 20 μl of either trypsin or chymotrypsin conjugated to agarose beads as a 1:1 slurry in buffer was added, and the tubes were mixed on a Nutator platform rocker. After 6 minutes, the trypsin sample was centrifuged to pellet the beads and the supernatant removed to a tube containing 3 μl of 1 mg/ml BPTI. The chymotrypsin sample was centrifuged after 10 minutes, and the supernatant added to 3 μl of 20 mg/ml TCPK. To these digests were added either empty procapsid shells, for a final concentration of 1 mg/ml (53 nM), or coat monomers to 0.5 mg/ml (10.5 μM). These reactions were incubated at room temperature for 2 hours. The samples were then centrifuged through 5-20% sucrose gradients for 35 minutes at 35,000 rev/min. 18 fractions were collected from each gradient through a pinhole at the bottom of the tube. The protein compositions of the fractions were determined by SDS-PAGE on 15% acrylamide gels.

**N-terminal sequencing**

Scaffolding fragments were generated with protease V8, trypsin, or chymotrypsin as described and separated by SDS-PAGE. The peptide fragments were transferred to polyvinylidine difluoride membranes (Immobilon PVDF, Millipore) according to the method of Matsudaira (1987). The transfer was performed in a Hoefer transfer unit at 100 mA for 16 hours at room temperature. The membrane was stained by Coomassie blue to reveal the location of the transferred peptide fragments. The 7 N-terminal residues

of each selected band were sequenced by the MIT Biopolymers Laboratory using an Applied Biosystems model 477A Perkin Sequencer with an on-line model 120 PTH Amino Acid Analyzer.

**Fluorescence and circular dichroism spectra**

Fluorescence spectra were recorded of wild-type or mutant scaffolding proteins at 100 μg/ml (0.3 μM) at constant temperature, with the excitation wavelength set to 280 nm, and the emission scanned from 300 to 400 nm. All other parameters were the same as above. Circular dichroism spectra were recorded from 200 to 250 nm at constant temperature in a 0.5 cm pathlength cell. The spectral bandwidth was 1.5 nm, the step size was 1 nm, the averaging time was 0.4 sec, and five scans were recorded and averaged for each sample.

**Fluorescence quenching**

KI quenching experiments were performed in phosphate/NaCl buffer, with 10 mM $Na_2S_2O_3$ added to keep the iodide reduced (Lakowicz, 1983). The final concentration of wild-type scaffolding protein was 100 μg/ml (0.3 μM); that of the two mutant proteins Y214W and Q149W was 50 μg/ml since the fluorescence of these mutants was approximately twice that of the wild-type protein. To avoid possible effects of ionic strength changes on protein conformation, the ionic strength was held constant by the addition of KCl, to give a total concentration of KI and KCl of 0.25 M. These concentrations of KI are sufficiently low so as to produce no inner filter effect (Teschke and King, 1993). Samples were excited at 280 nm and the emission was monitored at 330 nm. Excitation and emission slit widths were 2.5 and 5 nm, the PMT voltage was 700 and the temperature was 3°C.

**Thermal Denaturation**

Wild-type and mutant scaffolding proteins were diluted to 100 μg/ml (0.3 μM) in phosphate/NaCl buffer before use. Fresh samples were prepared for each experiment. Denaturation was monitored by either fluorescence intensity at 330 nm with excitation at 280 nm, or by circular dichroism ellipticity at 222 nm as described above for the GuHCl-induced denaturation.

For the fluorescence experiments the temperature of the controlling waterbath was adjusted in 2°C steps, from 3 to 89°C, and the sample equilibrated at each temperature until the reading was constant before recording the fluorescence intensity. For the circular dichroism experiments, the temperature was automatically adjusted in 1°C steps from 3 to 90°C with 2 minutes of equilibration at each point after reaching constant temperature. At the end of each experiment the sample was cooled back to 3°C to check for recovery of the original signal.

## GuHCl-induced denaturation of wild-type scaffolding protein

Low concentrations of the denaturant GuHCl are sufficient to extract scaffolding from procapsids, as discussed in Chapter 2. Since GuHCl is a denaturant, it is possible that it causes extraction by unfolding that part of the scaffolding molecule required for binding the coat shell. To determine if this might be the case, I studied the GuHCl-induced denaturation of purified scaffolding protein in order to see whether it was partially unfolded at the GuHCl concentrations that caused extraction.

Scaffolding protein was unfolded by incubation in concentrations of GuHCl from 0 to 4 M for at least 12 hours, after which time the samples have reached equilibrium and no further spectral changes occur. The folded state of the protein was estimated by two signals: fluorescence at 330 nm, which measures the solvent exposure of the single tryptophan residue, amino acid 134; and circular dichroism (CD) at 222 nm, a signal of alpha-helical structure. The experiments were carried out at 10°C, since studies of the thermal denaturation of scaffolding had indicated that unfolding began at temperatures as low as 15°C (M. Galisteo, unpublished results; this thesis). The concentration of scaffolding protein was 100 μg/ml, a concentration at which the protein should be almost entirely monomeric (Prevelige and Yphantis, unpublished results). Dilution of the protein out of GuHCl led to complete recovery of the original signal, demonstrating that the GuHCl-induced unfolding is reversible.

As shown in Fig. 4.2, the loss of secondary structure began upon the addition of minimal amounts of denaturant. The transition monitored by the fluorescence signal was not coincident with that observed by CD, indicating

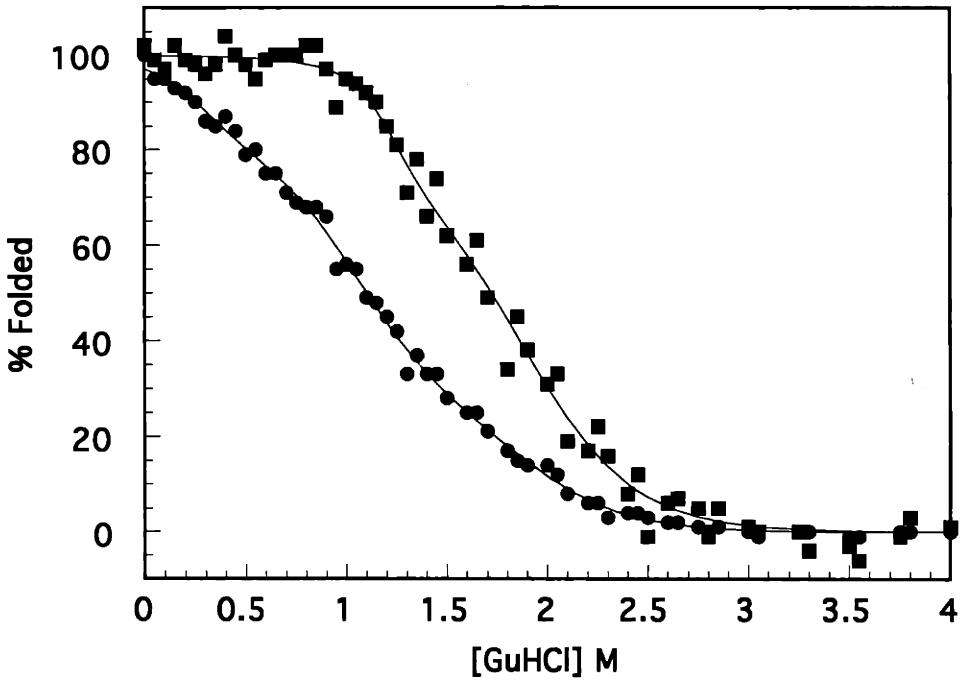



FIG. 4.2 GuHCl-induced unfolding of wild-type scaffolding protein. Wild-type scaffolding protein was diluted to 100 μg/ml (0.3 μM) in varied concentrations of buffered GuHCl. When equilibrium was reached after incubation overnight, the percent folded was determined as described in Materials and Methods from the fluorescence emission at 330 nm (squares) when excited at 280 nm, and the circular dichroism ellipticity at 222 nm (circles). Both curves include data points from three independent experiments. The solid lines are fits to to equations representing the sum of two (fluorescence) or three (circular dichroism) two-state transitions.

that the unfolding process was not two-state. The fluorescence transition did not begin until GuHCl concentrations at which about 40% of the secondary structure had already been lost. As the fluorescence signal is due to a single tryptophan residue, one explanation is that the fluorescence signal monitors the unfolding of a particular folding domain containing the tryptophan that is more stable than other regions of the protein.The fluorescence data could be fit as the sum of two transitions with midpoints at 1.2 and 1.7 M GuHCl, while the CD data displayed three transitions, with midpoints at 0.3, 1.1 and 1.9 M GuHCl. Thus the CD signal may in fact reveal the unfolding of a particularly unstable domain. Since a GuHCl concentration of only 0.5 M is sufficient to extract all the scaffolding from the coat shells (see Fig. 2.2), this could be the domain required for mediating binding of scaffolding to the coat shell. This domain presumably does not include the region surrounding the tryptophan, at residue 134.

**Proteolytic digestion of folded and partially unfolded scaffolding**

The GuHCl-induced unfolding of scaffolding protein was not a two-state process, suggesting the possibility of more than one folding domain. I was particularly interested in the region of the molecule that unfolded at low GuCHl concentrations, since this appears to be a region required for binding to the coat shell. The regions of ribonuclease A affected by partial thermal denaturation were identified by determining which parts of the protein became accessible to proteases at high temperature. (Rupley and Scheraga, 1963; Ooi et al., 1963) In order to determine which part of the scaffolding protein unfolded first, I looked for regions of the molecule that became more accessible to protease V8 after incubation in low concentrations of GuHCl at

10°C. Protease V8 was chosen since it is resistant to denaturation by GuHCl (Bond, 1993).

The protease digestion patterns of native and partially unfolded scaffolding are shown in Fig. 4.3. Under the buffer conditions used, protease V8 cleaves after both glutamic and aspartic acid residues (Houmard and Drapeau, 1972). As the amount of GuHCl is increased, the pattern of cleavage changes, with new bands becoming more prominent. The action of the protease is also inhibited, requiring longer digestion times, but this inhibition is due to the chloride ions, which inhibit V8, not the guanidinium. As shown in the last two lanes of Fig. 4.3, the presence of 1.5 M NaCl has the same inhibitory effect as the GuHCl, but the pattern of cleavages is not altered, demonstrating that the exposure of new cleavage sites is indeed due to denaturation of the scaffolding protein by GuHCl.

The major cleavage bands for both folded and partially unfolded scaffolding were blotted onto PVDF membranes and their N-termini were sequenced to determine the location of the cleavage sites. The size of each fragment was calculated based on a calibration with a set of molecular weight standards, so that in cases where the cleavage was in the C-terminus, the cleavage site could be estimated based on fragment size. The fragments that were sequenced, and their determined or estimated cleavage sites, are shown in Table 4.1. The three new sites exposed by GuHCl denaturation were all in the C-terminal half of the molecule, indicating that a C-terminal region unfolds first. This result is consistent with the location of the tryptophan residue in the N-terminal half of the protein.

FIG. 4.3 V8 proteolysis of native or partially denatured scaffolding protein. Scaffolding protein at 0.6 mg/ml was incubated with protease V8 at 10°C in buffer or buffer with GuHCl (or NaCl) added as described in Materials and Methods. After the times indicated the reactions were stopped and the samples were analysed by SDS-PAGE.

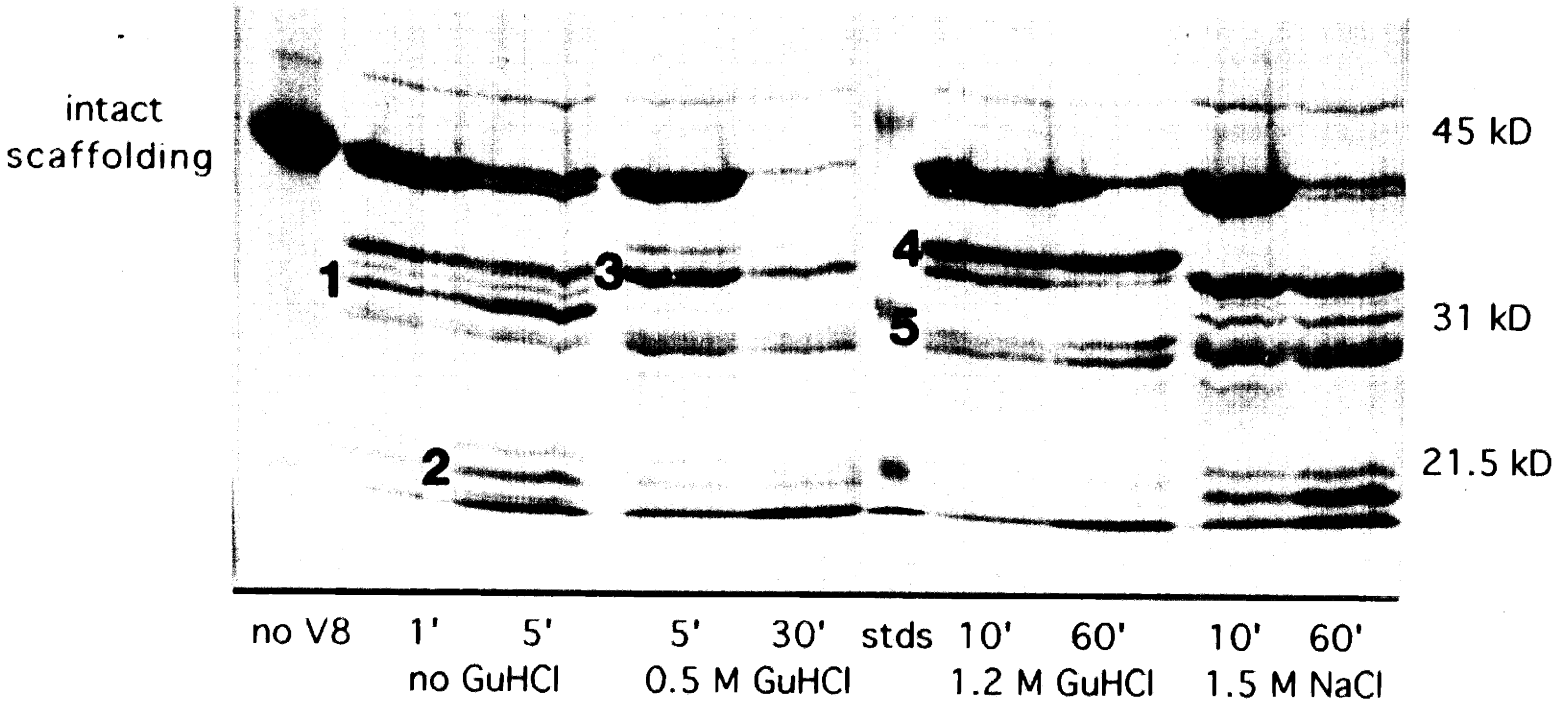

intact
scaffolding
45 kD
31 kD
21.5 kD
1
2
3
4
5
no V8
1'
5'
5'
30'
stds
10'
60'
10'
60'
no GuHCl
0.5 M GuHCl
1.2 M GuHCl
1.5 M NaCl

TABLE 4.1
Protease V8 cleavage sites in the presence and absence of GuHCl

| Fragment | Size | [GuHCl] | Cleavage site |
|---|---|---|---|
| 1 | 31.5 kD | 0 M | E62 |
| 2 | 19.5 kD | 0M | E155 |
| 3 | 34 kD | 0M and 1.2 M | E40, E43 |
| 4 | 35 kD | 1.2 M | D267* |
| 5 | 28.5 kD | 1.2 M | E40 and D261* |
| | | | E221* |

* Cleavage site estimated based on fragment size

**Function of proteolytic fragments**

In order to test the assignment of the C-terminus as the essential end for binding, I decided to make proteolytic scaffolding fragments and assay them for activity. Fragments were generated with either trypsin or chymotrypsin rather than V8, since inhibitors were available to stop the proteolysis without denaturing the scaffolding. Samples of scaffolding protein were digested for a given amount of time with each protease, before addition of the appropriate inhibitor to stop the proteolysis. The entire digest was then mixed either with purifed empty coat shells, to test whether the fragments could enter and stably bind, or to monomeric coat protein subunits, to test whether fragments could be incorporated into assembling procapsids. The reactions were allowed to proceed for two hours, then centrifuged through sucrose gradients to separate scaffolding fragments stably associated with capsids from those remaining as monomers.

Digestion of scaffolding by chymotrypsin resulted in only one cleavage, generating a large fragment of 30 kD and a small one at 10 kD. The large chymotryptic fragment was active in both assays (Figs. 4.4b and 4.5b), binding

FIG. 4.4 Binding of scaffolding fragments to empty procapsid shells. Scaffolding protein at 1 mg/ml (3 μM) was digested by either (a) trypsin or (b) chymotrypsin as described in Materials and Methods. Empty procapsid shells were added to 200 μl of each digest for a final concentration of 1 mg/ml (53 nM). The reactions were incubated at room temperature for 2 hours, then centrifuged through 5-20% sucrose gradients for 35 minutes at 35,000 rev/min. 18 fractions were collected from each gradient through a pinhole at the bottom of the tube. The fractions were run on 15% SDS gels and the gels stained by Coomassie blue.

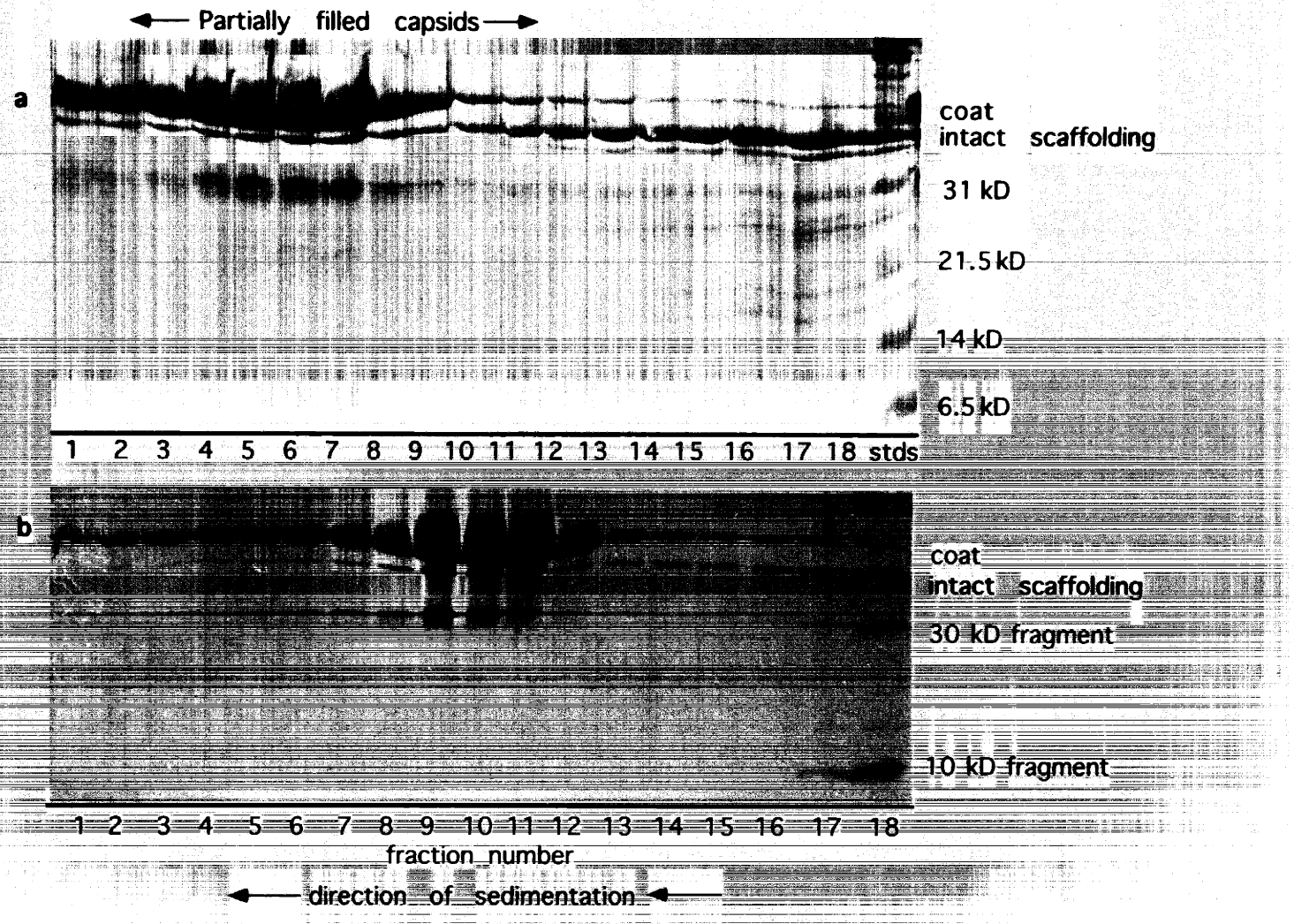
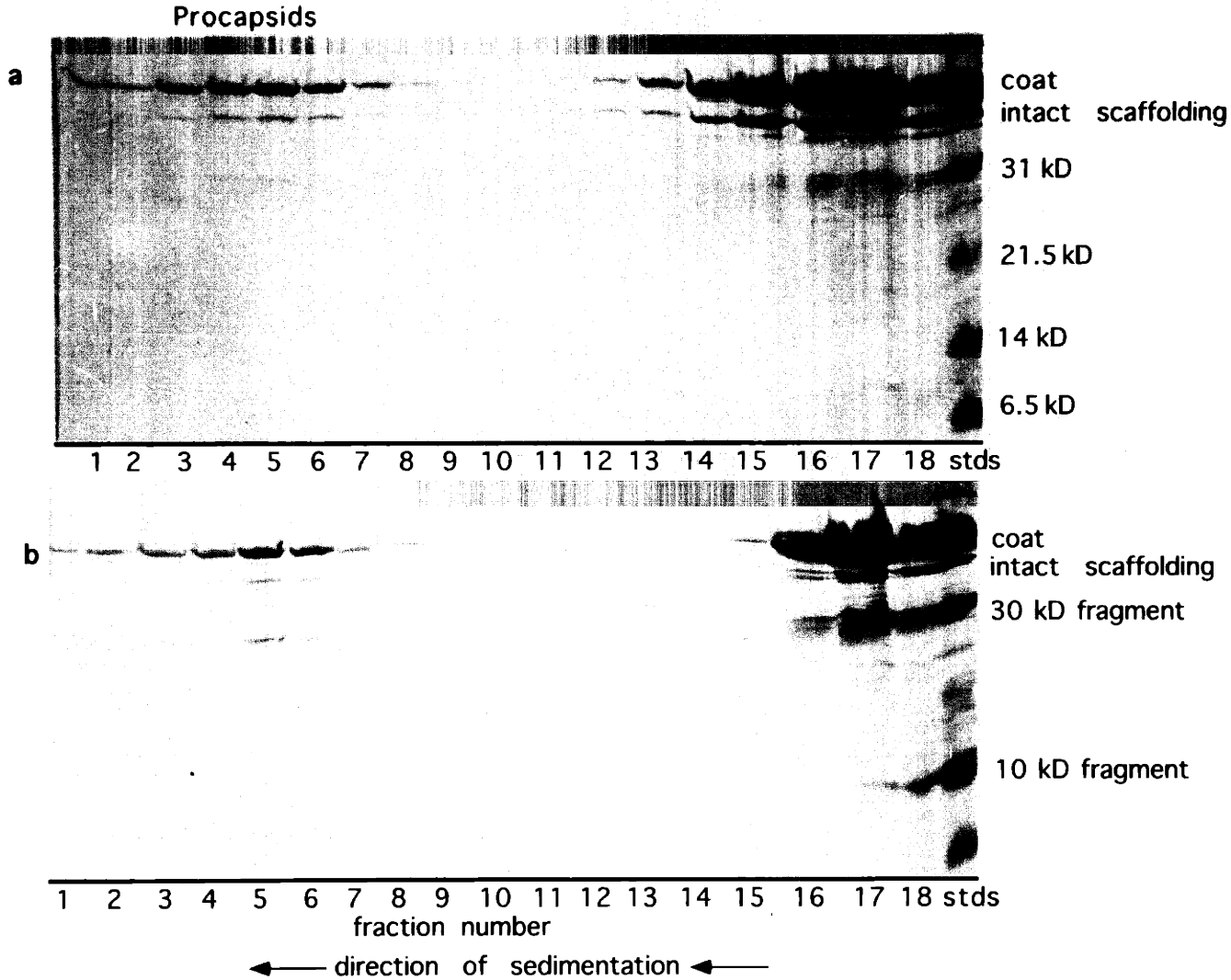

Partially filled capsids
a
coat
intact scaffolding
31 kD
21.5 kD
14 kD
6.5 kD
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 stds
b
coat
intact scaffolding
30 kD fragment
10 kD fragment
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
fraction number
direction of sedimentation

FIG. 4.5 *In vitro* assembly of scaffolding fragments into procapsids. Scaffolding protein at 1 mg/ml (3 μM) that had been digested by either (a)trypsin or (b) chymotrypsin as described in Materials and Methods. Coat monomers were added to 200 μl of each digest to give final coat protein concentration of 0.5 mg/ml (10.5 μM). The reactions were incubated at room temperature for 2 hours, then centrifuged through 5-20% sucrose gradients for 35 minutes at 35,000 rev/min. 18 fractions were collected from each gradient through a pinhole at the bottom of the tube. The fractions were run on 15% SDS gels. The gels were stained by Coomassie blue.


Procapsids
a
coat
intact scaffolding
31 kD
21.5 kD
14 kD
6.5 kD
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 stds
b
coat
intact scaffolding
30 kD fragment
10 kD fragment
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 stds
fraction number
direction of sedimentation

to the coat shells and incorporation into assembling capsids. The small fragment did not associate with the capsids under any conditions.

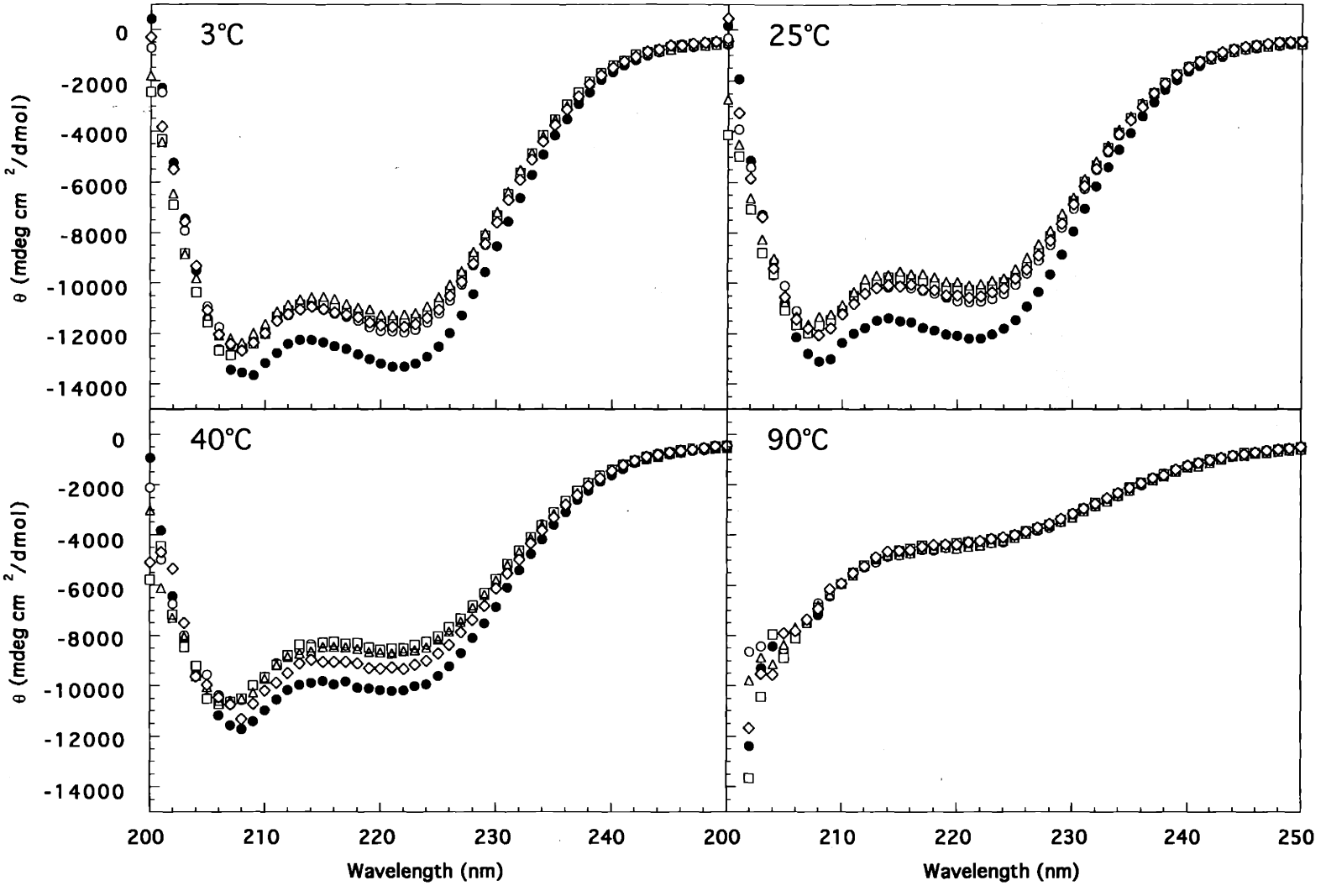
Trypsin digestion of scaffolding resulted in a range of bands, from approximately 35 kD to 10 kD. The bands from the trypsin digest migrating at 30 kD were able to associate with the coat shells in both assays, while a larger band migrating at 35 kD was not active in either assay (Figs. 4.4a and 4.5a). The 30 kD chymotrypsin band, and the 30 kD and 35 kD trypsin bands were chosen for N-terminal sequencing. Both the 30 kD bands had lost their N-termini, from cleavages at Y63 and R65. The 35 kD fragment had an intact N-terminus, and was estimated to have lost approximately 20-30 residues from the C-terminus. These results demonstrate that the scaffolding C-terminus is essential for any interaction with the coat protein, while an N-terminal region of at least 65 residues is dispensible, at least in the presence of full-length scaffolding.

**Structure of the mutant scaffolding proteins**

In the previous chapter I showed that none of the scaffolding mutations prevented the scaffolding protein from associating with the coat protein. This may be explained by their location outside the extreme C-terminus. I wished to study the folding of these mutants in the hopes of identifying additional structural/functional domains. As four of the mutants have a temperature-sensitive phenotype, I chose to examine their properties as a function of temperature rather than GuHCl concentration, in the belief that this data would have greater physiological relevance.

The fluorescence and CD spectra of the mutant proteins were first compared to that of wild-type to determine whether they folded to similar conformations. Fig. 4.6 shows the CD spectra of the wild-type and mutant

FIG. 4.6 Secondary structure of wild-type and mutant scaffolding proteins at different temperatures monitored by circular dichroism. WT = filled circles, S242F = empty circles, Y214W = squares, L177I = triangles, Q149W = diamonds. The protein concentration was 100 μg/ml.


3°C
25°C
40°C
90°C
θ (mdeg cm ²/dmol)
0
-2000
-4000
-6000
-8000
-10000
-12000
-14000
200
210
220
230
240
250
Wavelength (nm)

scaffoldings at several temperatures. As expected, the mutant proteins were, like wild-type, predominantly alpha-helical. Even at 3°C however, at which point all the proteins should be maximally folded, the mutant proteins all had different CD spectra than wild-type.

The secondary structure compositions of the folded proteins were estimated using the Chang, Wu, and Yang standards (Chang et al., 1978). Analysis of the wild-type CD spectrum suggested a composition of 30% alpha helix, 10% beta sheet, 18% turn, and 42% random coil, in excellent agreement with the composition determined by Teschke et al. (1993). The spectrum did not display the high $\theta_{222}/\theta_{208}$ ratio typical of a coiled-coil, but was characteristic of a single helix (Zhou et al., 1992). The spectra of the four mutant proteins at 3°C were similar to each other, and were composed of 25% alpha helix, 10% beta sheet, 20% turn, and 45% random coil. The pattern of secondary structure alteration for the mutants resembles that observed for the wild-type protein at higher temperatures. The spectrum of wild-type scaffolding protein at 25°C may be fit as 27% alpha helix, 10% beta sheet, 19% turn, and 44% random coil. These results suggest that the mutants are in a similar conformation at 3°C to that adopted by the wild-type protein at higher temperatures. By 90°C the spectra of the wild-type and the four mutant proteins were superimposible, and predominantly random coil, demonstrating that all had reached the same unfolded state.

The fluorescence of the two mutants without extra tryptophans were similar to wild-type over a range of temperatures; L177I was almost identical, while the fluorescence of S242F was slightly less, perhaps indicating a slightly less compact structure (Fig. 4.7). The fluorescence of both the mutant proteins containing extra tryptophans, Y214W and Q149W, was approximately twice that of wild-type, as expected. The spectra of these two mutants appeared

slightly red-shifted with respect to wild-type, suggesting that the new tryptophans might be in more exposed environments than the wild-type tryptophan.

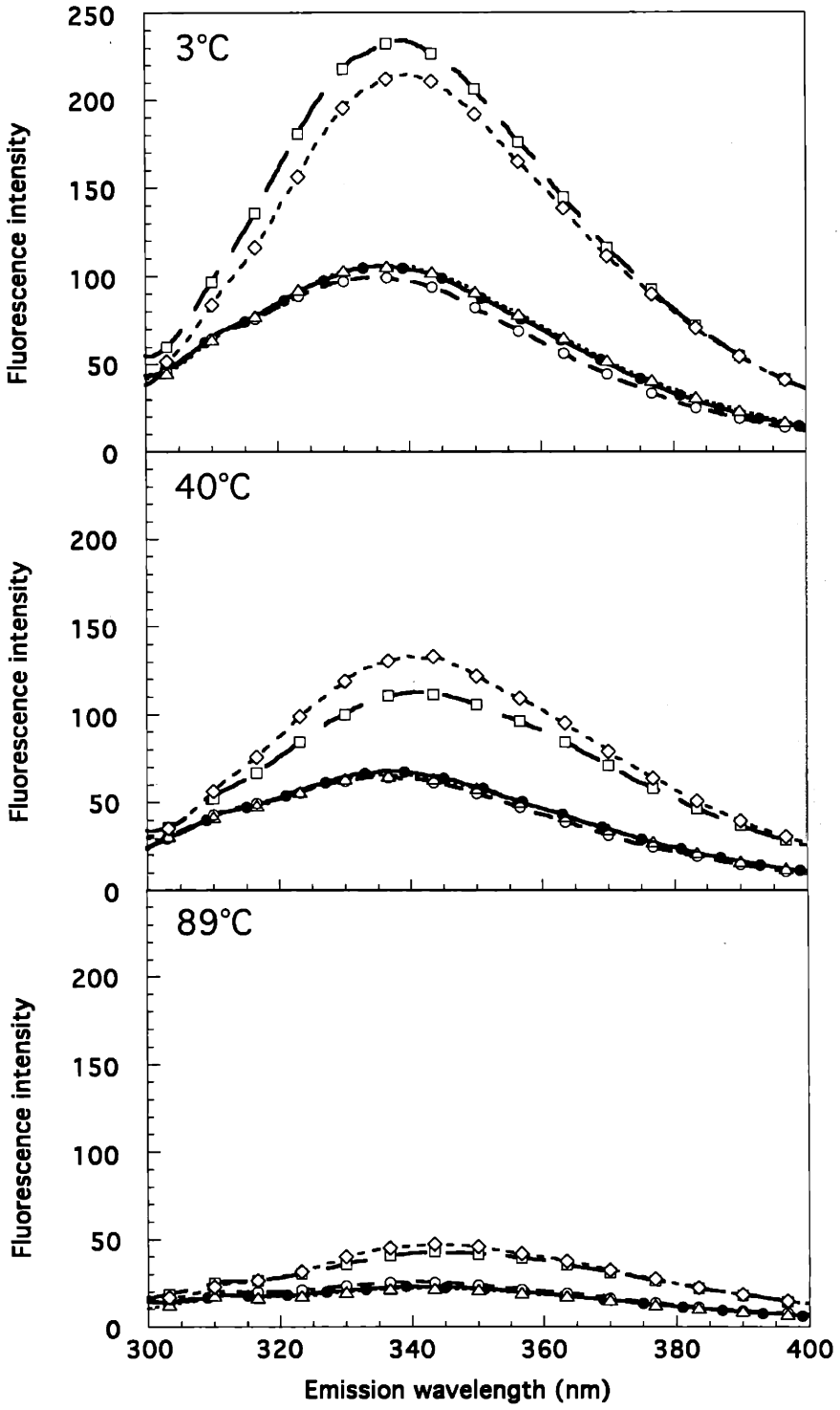
The degree of tryptophan exposure can be assessed by collisional quenching of fluorescence using iodide ion. Since iodide is an ionic molecule it cannot penetrate into protein interiors, so more deeply buried tryptophans are less effectively quenched. This experiment was carried out at 3°C, at which point the proteins would be maximally folded.

A Stern-Volmer plot (Lakowicz, 1983) of the fluorescence quenching by iodide of wild-type and the two mutant scaffoldings is shown in Figure 4.8. The slope of the line for each protein indicates the degree of quenching; the steeper the slope, the greater the degree of quenching. The wild-type tryptophan is least quenched. The slope of its line is about 1, which indicates little quenching, as expected for a tryptophan mostly buried in the protein interior. The slopes for the mutant proteins' fluorescence are clearly larger, although the data for the mutant proteins may not be linear, but reflect the presence of two fluorophores with different accessibilities. The slopes for the mutant proteins are approximately 1.5 for Q149W and 2.2 for Y214W. The tryptophan at 149 is thus more exposed than that at 134, but may still be partially buried, while that at 214 is probably on or near the surface of the protein.

**Thermal denaturation of wild-type scaffolding**

The thermal denaturation of the wild-type scaffolding (Fig. 4.9) gave similar results to the GuHCl denaturation. The unfolding curves measured by CD and by fluorescence did not coincide, demonstrating once again that the unfolding process is not two state, and consistent with the presence of more

FIG. 4.7 Fluorescence emission spectra of wild-type and mutant scaffolding proteins at different temperatures. WT = filled circles, S242F = empty circles, Y214W = squares, L177I = triangles, Q149W = diamonds. The protein concentration was 100 μg/ml. The excitation wavelength was 280 nm.


3°C
40°C
89°C
Fluorescence intensity
Fluorescence intensity
Fluorescence intensity
250
200
150
100
50
0
300
320
340
360
380
400
Emission wavelength (nm)

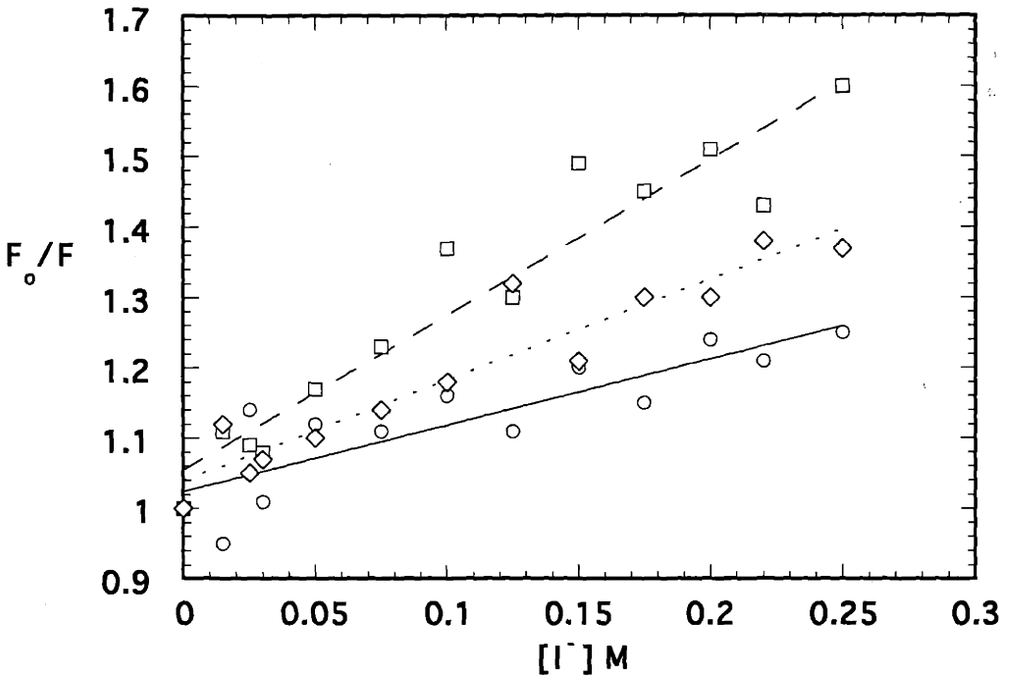



FIG. 4.8 Stern-Volmer plot for the quenching of wild-type and mutant tryptophans by iodide. Wild-type scaffolding protein at 100 μg/ml, or Y214W or Q149W mutant scaffolding protein at 50 μg/ml was incubated in increasing concentrations of KI. The fluorescence emission at 330 was measured with the excitation at 280 nm. $F_o$ is the fluorescence in the absence of iodide and F is the fluorescence in the presence of iodide. Circles = wild-type, squares = Y214W, diamonds = Q149W.

than one domain. The fluorescence transition was shifted to higher temperatures than that seen by CD, confirming that the region containing the single wild-type tryptophan residue is a more stable part of the protein. The CD transition was unusually broad, occurring over a temperature range of 60°C. This suggests that unfolding is either quite uncooperative, or involves multiple unfolding domains. The wild-type scaffolding was also a very unstable protein. Scaffolding protein began to denature at 15°C, and had lost about 13% of its secondary structure by 30°C, the normal temperature of growth *in vivo*.

In contrast to the GuHCl-induced unfolding, the thermal denaturation was not fully reversible, as only 80-90% of the original signal was recovered after cooling. The protein did not appear to form large aggregates since the light scattering did not increase significantly. Some of the protein may be irreversibly damaged by long periods at high temperature.

The denaturation curves were fit as either a single two-state transition or the sum of two or three such transitions. The fluorescence data is not well fit as a single transition, but appeared to be the sum of two transitions, one with a $T_m$ of 53°C responsible for 80% of the signal and one of smaller amplitude with a $T_m$ of 71°C. These correspond in amplitudes to the two transitions observed in the GuHCl-induced denaturation. The CD data could be well fit as a single two-state transition, although this process must actually include the two events observed by fluorescence as well as an additional event at lower temperature. It was not possible to fit the CD data so as to give transitions with the same midpoints as found for the fluorescence data, suggesting that the process may involve more than three transitions.

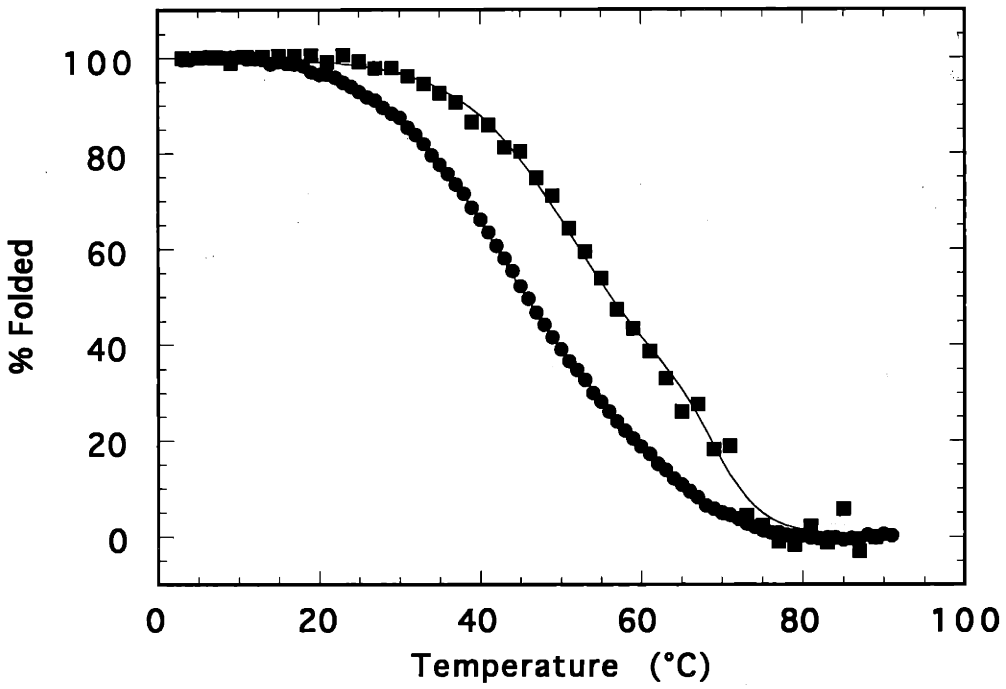



FIG. 4.9 Thermal denaturation of wild-type scaffolding protein. Wild-type scaffolding protein at 100 μg/ml (0.3 μM) was heated from 3 to 90°C, and the unfolding process monitored by fluorescence emission at 330 nm (squares) with the excitation at 280, or by circular dichroism at 222 nm (circles). The percentage folded was calculated as described in Materials and Methods. The solid lines are fits to equations representing the sum of two two-state transitions.

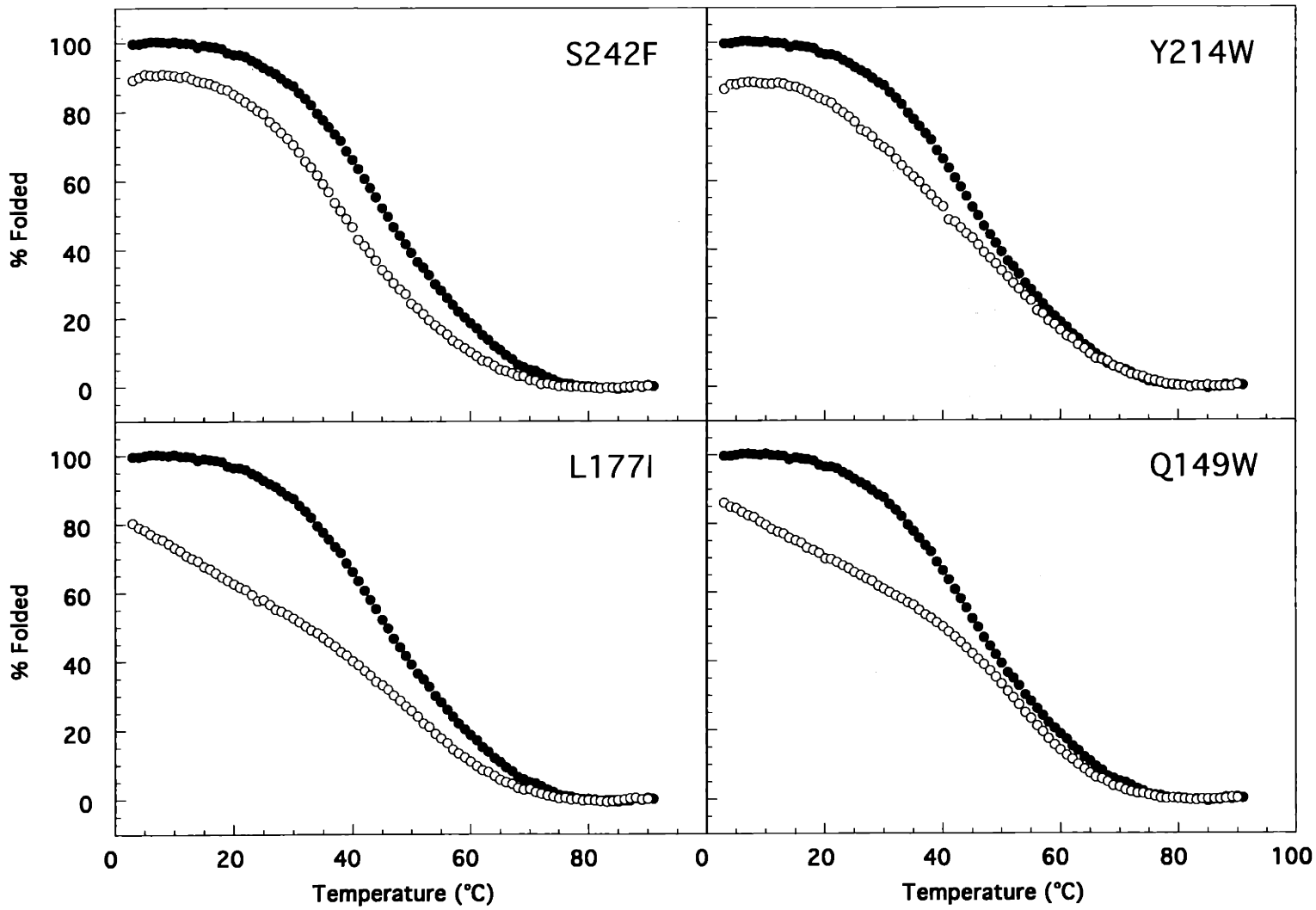
**Thermal denaturation of mutant scaffolding proteins**

Thermal melts of all the mutant proteins were performed as for wild-type. As monitored by CD at 222 nm all the mutant proteins were at least partially destabilized with respect to wild-type (Fig. 4.10). The mutant curves were normalized to start at points lower than the wild-type baseline since their CD spectra at 3°C (Fig. 4.6) all indicated that the mutant proteins began with less secondary structure than the wild-type protein.

The thermal denaturations of the mutant proteins S242F and Y214W had similar characteristics. While these proteins were initially less stuctured than wild-type, they appeared to have folded baselines at low temperture. The unfolding transition for both these mutants began earlier than for wild-type. Unlike wild-type, the data for these mutants were not well fit by a two-state model, indicating that one or more domains was more strongly affected than others. This effect was more visible for Y214W, for which the early part of the denaturation curve is shifted to lower temperature while the later half of the curve corresponds closely to the wild-type curve. The mutant S242F appeared more uniformly destabilized.

The Q149W and L177I mutations produced more dramatic destabilizations. The thermal melts of these mutant proteins were clearly not two-state, but showed a sharp break between two processes at about 40°C. The first region, containing approximately half of the total secondary structure, was extremely unstable, as it began to denature at the lowest temperature. This melting of this region appeared very non-cooperative, with the transition being almost flat, rather than sigmoidal. Indeed, this phase resembled the melting of a molten globule more than a folded protein. The rest of the molecule, however, was as stable as wild-type in the case of Q149W and only slightly destabilised for L177I. While this data was best described as

FIG. 4.10 Thermal denaturation of mutant scaffolding proteins. Mutant scaffolding proteins at 100 μg/ml were heated from 3 to 90°C and their denaturation followed by circular dichroism ellipticity at 222 nm. The percentage folded at each temperature was determined as described in Materials and Methods. The thermal melt of wild-type protein (filled circles) is shown for comparision to the mutant protein (open circles) in each case.


S242F
Y214W
L177I
Q149W
% Folded
% Folded
100
80
60
40
20
0
0
20
40
60
80
100
Temperature (°C)
Temperature (°C)

the sum of three two-state transitions, even this fit showed discrepancies with the data at low temperatures. This may be because the actual thermal melt involves four or more transitions, or because the denaturation events at low temperature did not represent a cooperative process.

**Unfolding of new domains observed by fluorescence of extra tryptophans**

The thermal melts of the mutant proteins were also observed by fluorescence. Two mutant proteins, S242F and L177I, contained only the single wild-type tryptophan residue. As observed by fluorescence, their thermal melts were similar in shape to that of wild-type although somewhat destabilized, S242F more than L177I, as expected from the CD data. These data could also be fit as the sum of two transitions, both of which had midpoints at lower temperatures than wild-type (Table 4.2). The fluorescence data confirmed that the more stable Trp-containing region was not seriously affected by the L177I substitution, despite the severe destabilization of secondary structure observed by CD. The region affected must be the C-terminal half of the protein.

The substitutions which introduce extra trytophans would be expected to reveal new phases in unfolding if the tryptophans are in different domains than the wild-type tryptophan residue. Both Y214W and Q149W did give melting curves that differed in shape from wild-type. The CD data served as an essential control for interpreting these differences. As the CD curves of Y214W and Q149W scaffolding proteins were very similar to those of S242F and L177I respectively, the differences seen between these pairs by fluorescence must be due to the presence of extra tryptophans, not to the destabilizing effects of the mutations. The Y124W and Q149W fluorescence curves could be fit as the sum of three transitions, two of which corresponded

TABLE 4.2

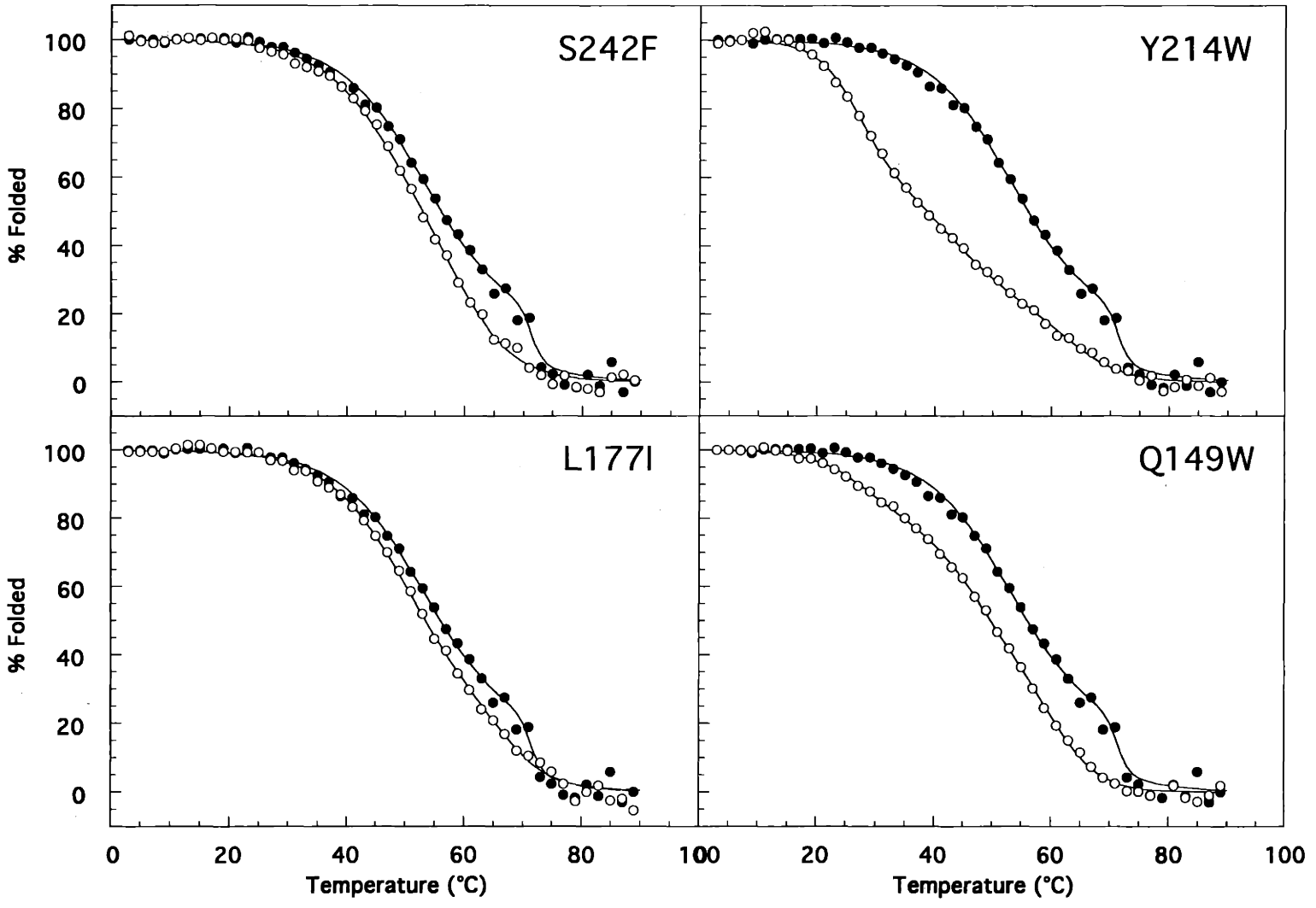
Calculated $T_m$s for denaturation transitions observed by fluorescence

| Mutant protein | $T_m$ 1 (°C) | $T_m$ 2 (°C) | $T_m$ 3 (°C) |
|---|---|---|---|
| Wild-type | | 53.3 | 71.3 |
| S242F | | 48.3 | 59.5 |
| Y214W | 29.7 | 52.0 | 68.5 |
| L177I | | 50.6 | 67.3 |
| Q149W | 34.6 | 53.6 | 65.3 |

to the wild-type transitions (Table 4.2), and a new one at lower temperature.

Surprisingly, the early unfolding event seen by CD in the thermal melt of Q149W was not observed by fluorescence, implying that the first broad phase observed by CD might involve two events. The first part of the protein to unfold in this mutant may not be the site of the mutation itself, but a region with which this site interacts, probably the more C-terminal sequence.

FIG. 4.11 Thermal denaturation of mutant scaffolding proteins monitored by fluorescence. Mutant scaffolding proteins at 100 μg/ml were heated from 3 to 89°C and their denaturation followed by fluorescence at 333 nm with excitation at 280 nm. The percentage folded at each temperature was determined as described in Materials and Methods. The thermal melt of wild-type protein (filled circles) is shown for comparision to the mutant protein (open circles) in each case. The solid lines are fits to equations representing the sum of two (WT, S242F, L177I) or three (Y214W, Q149W) two-state transitions.


S242F
Y214W
L177I
Q149W
% Folded
% Folded
Temperature (°C)
Temperature (°C)
0
20
40
60
80
100

**Scaffolding protein contains multiple domains**

The unfolding of P22 scaffolding protein to an apparent equilibrium by both GuHCl and heat revealed a transition that was definitely not two-state since two signals, fluorescence and CD, failed to give coincident transitions. In both cases, the transition monitored by fluorescence lagged behind that monitored by CD. Since the CD signal is due to secondary structure, in this case alpha helix, while the fluorescence signal indicates compact tertiary structure, the fluorescence signal would be expected to drop first if scaffolding denaturation proceeded through a molten globule intermediate (Ptitsyn, 1987). That some secondary structure was lost before any change in fluorescence was detected implies that the intermediates retained some tightly packed structure. These intermediates are therefore not molten globules but molecules in which only particular domains have unfolded.

Both the thermal and GuHCl-induced denaturation of wild-type scaffolding exhibited two cooperative transitions when monitored by fluorescence. There is only one tryptophan producing the signal, so the transition of larger amplitude probably results from unfolding of the domain actually containing the tryptophan while the later event is the unfolding of a nearby domain. Since the denaturation monitored by CD revealed additional unfolding occuring at earlier temperature, there must be at least three folding domains. This analysis was confirmed by the two mutant proteins containing extra tryptophans, both of which displayed an extra third transition by fluorescence. Given that the CD data for Q149W revealed denaturation at low temperature that was not detected by fluorescence, and that the Q149W and

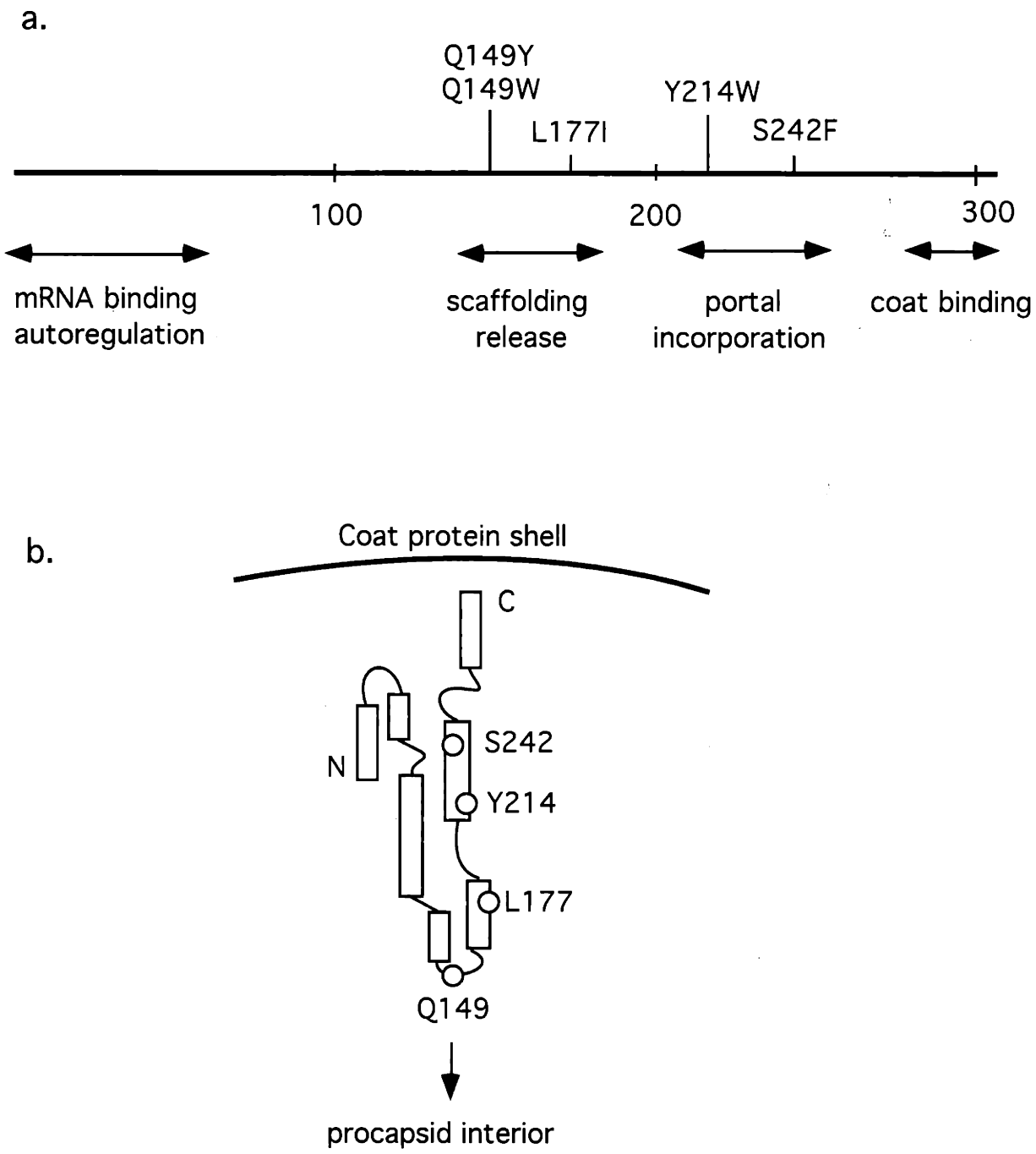



FIG. 4.12 Models for scaffolding protein structure

(a) Sites of functional regions along the scaffolding protein sequence

(b) A possible model of the scaffolding monomer

L177I CD data were not well fit even by three transitions, there may well be four or more structural domains. A model stucture is shown in Fig. 4.12.

**Identification of the coat-binding region**

Since low concentrations of GuHCl are sufficient to disrupt the binding interactions between scaffolding and coat shells, it is reasonable to conclude that the part of the molecule unfolded at these GuHCl concentrations is the region involved in binding. Identification of a region within the C-terminal half of the protein as the binding region was confirmed by the behaviour of proteolytic scaffolding fragments in the shell-binding and assembly assays, since loss of the C-terminal 20-30 residues made the scaffolding inactive, while loss of the 65 N-terminal residues had no effect.

Similar results have been obtained for other viral scaffoldings, as mentioned in the Introduction. A long amber mutant of the major scaffolding protein of bacteriophage T4, gp22, was able to assemble into naked cores *in vivo*. Although wild-type coat protein was present in these infections, it did not cover these cores, but formed polyheads as if scaffolding were absent (Traub and Maeder, 1984). The authors suggested that the T4 scaffolding consisted of two domains, of which the C-terminal domain was required for attachment of the core to the coat shell.

The herpesvirus scaffolding protein, VP22a undergoes a proteolytic cleavage that removes 25 amino acids from the C-terminus. When this cleavage is blocked by mutation, immature procapsids accumulate (Preston et al., 1983), suggesting that this cleavage is essential for release of the scaffolding. Assembly of herpesvirus procapsids requires the C-terminal 25 amino acids of either the scaffolding or its protease, which includes the scaffolding sequence within its coding region. When only truncated versions

of the two proteins were available, aberrant capsids were formed as in the complete absence of the proteins (Thomsen et al., 1995; Matusick-Kumar et al., 1995). An essential coat-binding tail at the scaffolding C-terminus may be a conserved feature amongst both phage and animal viruses.

**The portal-binding region**

The mutations S242F and Y214W block the incorporation of the DNA packaging portal into procapsids, indicating that these mutations affect a scaffolding site required either for binding the portal or forming an initiation complex to which the portal is recruited. Both mutations destabilized a C-terminal region, but S242F differed from Y214W in also affecting the stability of N-terminal domains. This difference may explain why S242F also blocks incorporation of the pilot proteins, while Y214W does not. While these mutations lie within the C-terminal region, they may interact with the N-terminal domains, so that the effects of the S242F mutation could be propagated into these domains. The N-terminus is involved in post-transcriptional repression of scaffolding synthesis (King et al., 1978; Casjens et al., 1985; Wyckoff et al., 1985), probably by binding to the mRNA near the site of its translational initiation. A model has been proposed in which a complex of scaffolding molecules bound to mRNA serves as the initiator of procapsid assembly (Bazinet et al., 1990). If this were so, then the portal-binding region would need to interact with the mRNA binding domain in order to insure that the portal was included in the initiating complex.

A portal binding region may also be conserved within other viruses. A missense mutation in the T4 major scaffolding protein, gp22, is supressed by mutations in the gene encoding the T4 portal protein (Mesyanzhinov et al.,

1990). This mutation is at at residue 172 of the 259 amino acid protein, approximately the equivalent region of the sequence as the P22 mutants.

**A scaffolding release domain**

The mutations at sites L177 and Q149 impair scaffolding release. As discussed in Chapter 3, there are several ways to interpret this effect. The mutations may be at a site which binds to the coat shell, and alter the binding surface so that it is "stickier" at high temperature, thus blocking release. That these mutant sites are part of a coat-binding region seems less likely given the identification of the extreme C-terminus as an essential coat-binding region, although it is possible that these two regions are nearby in the folded protein. Alternatively, the site of mutation may be a region involved in scaffolding/scaffolding binding. Again, by making this binding interaction stronger at high temperature, the mutant proteins could be blocked from exiting the capsid, since they must do so as monomers, given the size of the exit channels. A different possibility is that this region actively recognizes that the DNA has begun to enter, by sensing either the DNA itself or changes in the portal induced by packaging initiation, and signals the other scaffoldings to alter conformation and be released. By destabilizing this region at high temperature, the mutations would make the scaffolding unable either to recognize or respond to the release signal.

If the mutations increased the affinity of a binding surface, they would not be expected to have much effect on protein stability, as they would be on the outer surface of the protein. The mutations might even act by stabilizing the domain involved in binding, maintaining it in the correct conformation required for the interaction and decreasing the entropic cost of binding.

The actual effect of the mutations was to significantly decrease the stability of a large portion of secondary structure. It is possible that the destabilization of this domain could lead to aberrant aggregation of the scaffolding subunits that would trap them inside the procapsid. Scaffolding constructs which lack this region, however, appear to also be defective in release *in vivo* (discussed below). Taken together these results are most consistent with the idea that the mutations alter a domain with a specific active function, that of a scaffolding "release switch," that it can no longer perform.

The loss of secondary structure in these two mutant proteins began at lower temperature than the loss of fluorescence, as monitored by the new tryptophan residue substituted for Q149. This secondary structure may not be part of the region including the mutations Q149W and L177I, but a separate domain with which it interacts. This domain is probably the C-terminus, as the N-terminus appears not to be significantly destabilized. An interaction with the C-terminus could be relevant *in vivo* as part of the mechanism for inducing scaffolding release.

**Mechanism of the Q149W assembly defect and cold-sensitive phenotype**

In addition to its defect in scaffolding release, the mutant Q149W causes assembly of many aberrantly shaped capsids at high temperature, a property not shared by L177I. There is, however, no significant difference in the thermal denaturation of these two mutants that would explain why Q149W has additional defects *in vivo*; in fact, Q149W is slightly more stable. This mutant also differs from L177I in possessing a cold-sensitive phenotype, resulting in accumulation of many empty procapsids at low temperature. The mutant protein displayed no sign of cold denaturation, but this was not

expected, as this phenomenon has only been observed under non-physiological conditions such as sub-zero temperatures or high concentrations of denaturants (Privalov et al., 1986; Griko and Privalov, 1992). Cold lability *in vivo* is thought to result not from protein denaturation but from weakened intersubunit interactions (Bock and Frieden, 1978).

The tryptophan at this site in the mutant protein was exposed to solvent, based on its accessibility to fluorescence quenching by iodide. (Fig. 4.8). While the large tryptophan sidechain may not have been able to pack in the same position as the glutamine, it is possible that the glutamine, a more polar residue than tryptophan, is also normally a surface residue. It may be involved in making an essential contact required during assembly. In this case the substitution of this residue could by itself cause defects apart from any resulting from destabilization of the domain. This could explain why the substitution Q149Y also gives a cold-sensitive, but not a temperature-sensitive phenotype.

**Functions of the stable N-terminal domains**

The N-terminal part of the scaffolding protein, up to at least the tryptophan at 134, contains two relatively stable domains. Unfortunately, there are no signals which monitor the unfolding of the extreme N-terminus. However, the more stable region of the protein includes at least half of the secondary structure, based on comparisions of the fluorescence and CD signals during unfolding of the wild-type protein, and the amplitude of the later phase observed in the denaturation of L177I and Q149W. For this reason I have assumed that the entire N-terminal 134 residues makes up the stable region.

As mentioned above, the scaffolding protein is a post-transcriptional regulator of its own synthesis (King et al., 1978; Wyckoff et al., 1985). Based on the behavior of scaffolding amber fragments the region up to residue 78 was determined to be sufficient for translational repression (Casjens et al., 1985). All three proteases used in these experiments cleaved the scaffolding between residues 60–65. This segment may be an exposed loop marking the end of an mRNA-binding domain consisting of the 60 N-terminal residues.

The other N-terminal domain would include the residues from 65 to at least 134. This region may have an as yet undetermined function, or it may simply serve to extend the protein's length. This region might need to be more stable so as to insure that the functional domains remained supported at the correct distances from each other.

**Studies of truncated scaffolding proteins**

Casjens and colleagues have studied the ability of altered constructs of scaffolding protein to rescue a scaffolding amber strain *in vivo*, and have characterized the structures produced (S. Casjens and L. Sampson, unpublished results). Their results support the conclusions described above. A construct lacking the 10 C-terminal amino acids was unable to assemble any procapsids, in accordance with my identification of the C-terminus as an essential coat-binding region. Constructs lacking either 140 or 180 amino acids were able to assemble procapsids that included the portal and minor proteins, confirming that regions C-terminal to the truncations included the portal-binding activity. This result also implied that mutations at 149 and 177 could not be part of an essential coat-binding region, since the construct lacking all 180 N-terminal residues could still assemble coat protein into procapsids. These procapsids were not, however, able to mature to phage, demonstrating

that they lack some function essential to DNA packaging and maturation. This is consistent with my proposal that the missing region is required to actively mediate scaffolding release.

**Why is the scaffolding protein so unstable?**

The P22 scaffolding is an unusually unstable protein, that begins to lose secondary structure upon addition of the smallest amount of denaturant, and at surprisingly low temperatures. The coat protein of P22 is equally unstable to denaturant (Teschke and King, 1993). It may be that unassembled viral proteins must be conformationally flexible in order to permit conformational changes required during the assembly process. The crystal structures of the RNA plant viruses (Harrison, 1984) as well as SV40 showed that the viral capsomeres are held together by entwined arms (Liddington et al., 1991). These arms could not have been folded before assembly, since they would not have been free to make such extensive interactions. A similar arm might attach the scaffolding to the coat protein shell.

Viral proteins might be similar to the small dimeric DNA binding proteins such as the P22 Arc repressor, whose crystal structure also reveals extensive intertwining of the two chains (Raumann et al., 1994). Arc does not exist in solution as a folded monomer (Bowie and Sauer, 1989; Milla and Sauer, 1994), probably because its structure is dependent upon interactions that can occur only within the dimer. The P22 coat and scaffolding proteins are both substantially larger proteins than the 53 amino acid Arc repressor, and unlike Arc, both probably have multiple domains. Thus some parts of the scaffolding might be quite stable, while the regions involved in binding interactions would lack a defined structure in the absence of binding. In particular, the scaffolding region that mediates binding to the coat shell

would need to be flexible since this interaction must be readily disrupted upon the initiation of DNA packaging.

Consistent with this analysis, the C-terminal regions of the scaffolding protein are the least stable. These regions appear to include all the functions required during assembly, including coat binding (the extreme C-terminus), portal binding (the sites of S242F and Y214W) and possibly scaffolding/scaffolding binding interactions (L177I and Q149W).

More quantitative determinations of the stabilities of different regions in multidomain proteins can be obtained by calorimetry (Privalov, 1982). When the scaffolding protein was studied by calorimetry, however, no thermal events were observed from 10 to 110°C (Galisteo and King, 1993). This result is surprising given that cooperative thermal events are observed by the spectroscopic signals. It is possible that the denatured protein in the calorimeter undergoes compensating interactions such as oligomerization, which could explain why the thermal denaturation is not completely reversible. The heat capacity change upon unfolding of globular proteins probably results from exposure of non-polar residues to aqueous solvent (Privalov, 1979; Privalov et al., 1989). The burial of surface by intermolecular interactions could compensate for the loss of intramolecular interactions, especially if the buried surface of the folded monomer is not large. Fibrillar proteins, which have a much higher surface to volume ratio, have significantly lower heat capacity changes upon unfolding (Privalov, 1982). These proteins typically have a fair amount of hydrophobic surface shielded by coiled-coil interactions; as the scaffolding protein does not show any signs of being a coiled-coil, it may be even more exposed. Consistent with a large exposure of non-polar residues, each scaffolding monomer binds 12-16

molecules of bis-ANS (Teschke et al., 1993), a small dye used to probe the accessibility of hydrophobic surfaces on proteins (Brand and Gohlke, 1972).

While these features are characteristic of a molten globule (Ptitsyn, 1990), the scaffolding protein does display what appears to be cooperative unfolding of distinct regions. The scaffolding protein simply may not have a hydrophobic core as for typical globular proteins. Perhaps it consists of a series of loosely interacting helices. Tight interactions of exposed helical surfaces are probably formed within the closely packed interior of the procapsid. The formation of these interactions might be a driving force in procapsid assembly. In this sense, the final product of the scaffolding folding pathway is not an individual scaffolding subunit, but an assembled procapsid.

It is interesting that the microtubule-binding protein tau is highly extended and contains almost no secondary structure in solution (Cleveland et al., 1977), leading to the suggestion that it exists in a "natively denatured" state (Schweers et al., 1994). The MAP-2 protein has similar properties (Voter and Erickson, 1982). While scaffolding protein does have substantial secondary structure, the proteins are similar in that all can function despite lacking the characteristics of folded proteins. The scaffolding protein may typify a new class of proteins that are not meant to lead an independent existence, and thus do not require the same degree of structure as a typical soluble enzyme.

# CHAPTER 5

# CONCLUDING DISCUSSION

The results of this thesis reveal a picture of the P22 scaffolding protein as a multidomain, multifunctional molecule, that interacts with the coat protein lattice in a complex manner. I will now consider some of the remaining fundamental questions about the scaffolding protein: How is it arranged within the procapsid, and by what mechanisms does it control assembly?

## STRUCTURE OF THE SCAFFOLDING PROTEIN WITHIN THE PROCAPSID

In order to understand the way in which scaffolding proteins guide the correct assembly of coat subunits, it would be extremely useful to observe the contacts made to the coat lattice within the assembled procapsid. Unfortunately, all the virus crystal structures solved to date have been mature virions. A few procapsid structures have been solved to around 30Å resolution by cryo-electron microscopy and image reconstruction, but these procapsids did not contain scaffolding protein.

The number of scaffolding molecules within a procapsid is approximately 270 (Casjens and King, 1974; Eppler et al., 1991), as compared to the 420 coat subunits that comprise the T=7 procapsid. This number of scaffolding subunits does not suggest any obvious integral relationship to the coat protein. This is consistent with the failure to detect small hetero-oligomers under a variety of conditions. The situation is further complicated by the fact that the 270 scaffolding molecules are proabably not all at equivalent positions within the procapsid. The experiments discussed in

Chapter 2, as well as the GuHCl extraction data for the mutant scaffoldings L177I and Q149W (Fig. 3.10) strongly suggests the existence of two classes of scaffolding subunits within procapsids. There may be two types of binding sites for scaffolding molecules within the shell lattice, or one class of molecules may not contact the coat protein at all, but bind to other scaffolding molecules.

In the discussion of Chapter 2, I considered how the number of specifically binding scaffolding subunits might relate to the icosahedral symmetry of the procapsid shell. Recent evidence, however, suggests that the scaffolding might not have the same icosahedral symmetry as the outer shell.

**Image reconstruction of the P22 scaffolding core**

The image reconstruction technique used to produce the structures of the procapsid and mature phage shown in Fig. 1.2 assumes that the particles possess icosahedral symmetry, and uses this symmetry to align the combined images (Crowther et al., 1970; Crowther, 1971). An advantage of this technique is that each particle contributes twenty views of the asymmetric unit, allowing reconstructions to be obtained from relatively few particles. The drawback is that features of the structure that do not possess icosahedral symmetry are not recovered. For instance, the portal structure present at a unique vertex is not visible in reconstructions of either procapsids or phage (Prasad et al., 1993), since its density is averaged out over all twelve vertices.

Attempts have been made by myself and others to reconstruct images of procapsids containing scaffolding protein (P. Thuman-Commike, B. Greene, J. Jakana, B. V. V. Prasad, P. Prevelige, J. King and W. Chiu, unpublished results). While some internal density was observed in the reconstructions, no consistent structure was produced, even though the

external shell of coat protein always appeared the same. This was the case even when the two reconstructions were done using particles from the same electron micrograph that had been processed together. It was possible that the procapsids within a given sample might be heterogeneous because they had lost varied amounts of scaffolding before the micrographs were taken. Yet procapsids containing the mutant scaffolding L177I, which is much more difficult to release from procapsids, gave no better results. This failure to obtain a consistent reconstruction is thus not due to inconsistencies in sample preparation or processing technique, but rather reflects some property of the scaffolding itself.

**Models for the arrangement of scaffolding subunits within procapsids**

Does the scaffolding core have any regular structure at all? Casjens and Hendrix (1988) have proposed that phage scaffoldings might direct assembly not by specific binding interactions but by forming a surface with complementary charged or hydrophobic features. Several phage scaffolding proteins, including that of P22, do have a non-random distribution of charges which might contribute to the formation of such a surface (Eppler et al., 1991). However, this model provides no indication as to why there would be two classes of scaffolding molecules. The rapid rate at which scaffolding subunits can reenter extracted coat shells (Fig. 2.6) also suggests some specific interactions.

The scaffolding may form a regular, symmetric structure that is non-icosahedral. The T4 scaffolding core appears to consist of six helical ribbons starting at the portal vertex (Paulson and Laemmli, 1976; Engel et al., 1982). The symmetry mismatch between the T4 shell and scaffold may used as a Vernier mechanism to set the T4 length (Paulson and Laemmli, 1976;

Kellenberger, 1990). This consideration does not apply to the icosahedral P22.

An intruiging variant on this theme was proposed by Casjens. In this model (Fig. 5.1a), the scaffolding molecules near the portal vertex are arranged radially, and make specific contacts with the coat shell. Those scaffolding subunits further from the initial vertex are shifted until they no longer make specific contacts to the coat. This model allows for the interaction of scaffolding with the portal, and also accounts for two classes of scaffolding subunits, with specific and non-specific binding. In capsids that have been partially refilled with scaffolding *in vitro*, the scaffolding does not appear to be concentrated at a single vertex, as this model would predict (B. Greene, unpublished results). However, artefacts of the negative stain technique may distort the appearance of the scaffolding; samples observed by cryo-electron microscopy might reveal such a distribution.

It is possible to devise models in which the scaffolding is arranged at icosahedrally symmetric points within the procapsid lattice, but is still not visible in reconstructed images. It may be that while the scaffolding subunits bind to specific sites, only a fraction of these sites can be occupied in any procapsid due to steric hindrance. While scaffolding molecules are always portrayed as if they extend radially inward, they may actually be somewhat tilted so as to block off more of the inner surface (Fig. 5.1b). Since the particular sites filled would vary randomly from procapsid to procapsid, the averaged density would not produce a consistent image. The two classes of scaffolding subunits would be those bound at specific sites, and those which have had to settle for squeezing in at random places.

Another possibility is that the scaffolding is dynamic, rather than fixed. While the scaffolding molecules do make specific contacts to the coat shell, these contacts might be sufficiently tenous that the rest of the molecule is free

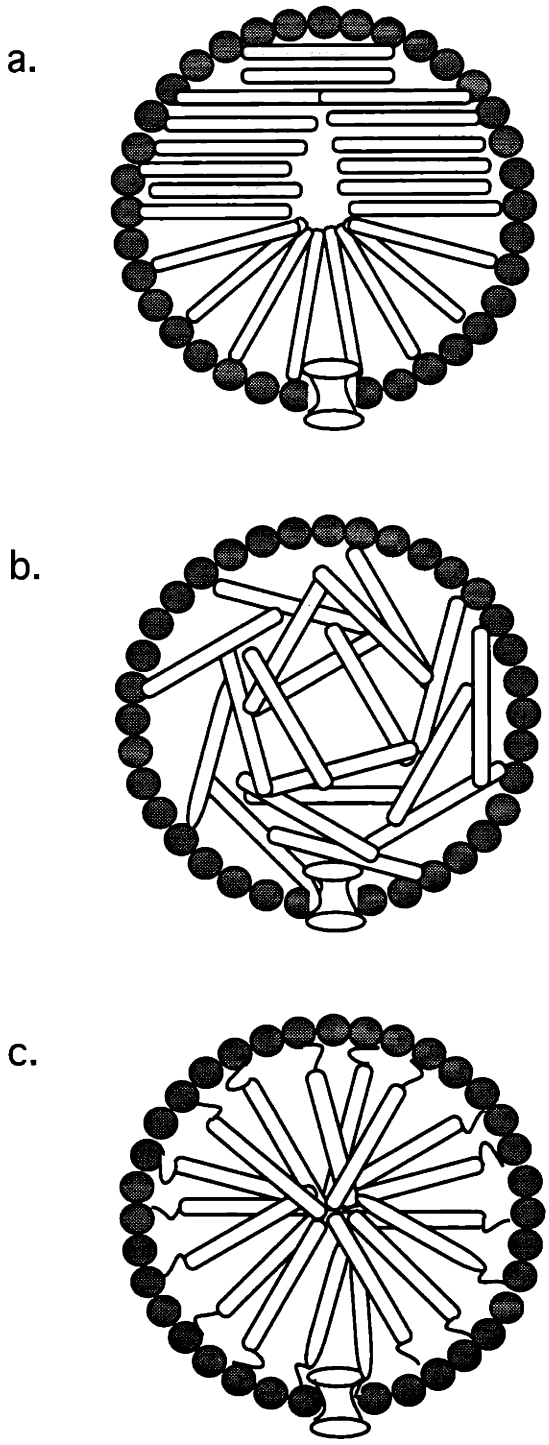



FIG.5.1 Models for the arrangement of scaffolding subunits within procapsids

to move within the shell, thus randomly blurring its position. In particles observed by negative stain, in thin sections, and as reconstructed images, there is a gap in density between the outer shell and inner core. The scaffolding might be connected to the coat by only a thin tether, that does not show up as density on the micrographs (Fig. 5.1c). Alternatively, it may be that immediately after assembly the scaffolding releases from the coat shell and is held within the shell solely by scaffolding/scaffolding interactions. In their "restless cable" model for the interaction of tropomyosin with the actin filament, Phillips and colleagues (Phillips et al., 1980; 1986) noted that although the long tropomyosin molecule has multiple sites for binding actin, disordered regions in the crystal structure suggested that the individual sites were not tightly bound, but were fluctuating in and out of contact with the filament. Once the scaffolding molecules have formed a core, the overall core surface would contain multiple binding sites and could perhaps interact with the inner capsid surface in a similar manner.

It may be relevant that procapsids made *in vitro* from coat and scaffolding subunits have a distinctly different morphology, lacking a defined central core but having thicker, fuzzier shells (Prevelige et al., 1988; Thomas and Prevelige, 1991). The *in vitro* assembly process may trap the capsid lattice in a precursor state not normally observed *in vivo.*, in which the scaffolding remains in tight contact with the coat shell. Image reconstructions of these particles are planned.

### HOW DOES SCAFFOLDING PROTEIN REGULATE ASSEMBLY OF THE COAT SUBUNITS?

I have identified a short (20–30 amino acid) region at the scaffolding C-terminus as essential for any interaction with the coat protein. When this region is removed, the truncated scaffolding protein is incapable of

assembling monomeric coat subunits into procapsids, or even binding to previously assembled empty procapsids. The role of the extreme C-terminus as a coat-binding segment appears to be conserved in other virus and phage scaffolding proteins. A long amber fragment of the T4 scaffolding protein forms naked cores that cannot interact with the coat protein (Traub and Maeder, 1984). Elimination of the C-terminal 25 amino acids from the HSV-1 scaffolding protein has a similar effect, as the shortened protein cannot prevent the HSV-1 capsid proteins from assembling aberrant structures (Thomsen et al, 1995; Matusick-Kumar et al., 1995), but is itself able to form cores *in vitro* (Newcomb and Brown, 1991) and *in vivo* (Kennard et al., 1995). Cleavage of this region is required *in vivo* for scaffolding release and DNA packaging (Preston et al., 1983).

The sequences of the phage scaffolding proteins reveal no discernible homology at the C-terminus to each other or to the scaffolding equivalents of herpesviruses. Both P22 and T4, however, have a non-random distribution of hydrophobic residues in their C-terminal 30 amino acids, as do the herpesvirus scaffoldings (Eppler et al., 1991; Marusich and Mesyanzhinov, 1989; McGeoch et al., 1988; Robson and Gibson, 1989). These regions have the potential to form amphiphilic alpha helices. Such helices might serve as a common motif for interaction with coat proteins.

A possible mechanism for how these C-terminal ends might regulate coat protein assembly is suggested by recent results on the binding of signal peptides to calmodulin (CaM). Calmodulin is a calcium regulatory protein that is activated by a number of enzymes, all of which share a common binding motif: a basic, amphiphilic alpha helix (O'Neil et al., 1990). In the absence of signal peptides CaM is an elongated protein consisting of two globular lobes separated by a long helical region (Babu et al., 1988). Peptide

binding induces a dramatic conformational change to a more compact structure. The conformation within the lobes is not significantly altered, but they are brought closer together by a large hinge movement in the central helix (Meador et al., 1992). Analysis of the binding interaction by NMR demonstrated that the signal peptide is unstructured in solution, but becomes helical upon binding CaM (Wand et al., 1995). The conformational change in CaM occurs after this step. The authors proposed that binding to CaM induces the peptide to adopt a helical conformation. This helix then serves as a surface on which to fold the CaM protein to its more compact state.

Scaffolding terminal helices could act in a similar fashion to induce hinge-bending in their coat proteins (Fig. 5.2). A CaM type of mechanism is appealing because it involves both a coat-induced conformational change in the scaffolding protein, which could allow the scaffolding to interact with other scaffolding or coat subunits, as well as a scaffolding-induced conformational change in the coat protein, to allow the coat to correctly associate with other coat subunits or to add the next scaffolding subunit. Some sort of mutually induced conformational change seems necessary since neither protein assembles efficiently on its own.

Hinge-bending motions in the coat protein were previously proposed in order to explain the maturation expansion of P22 and other phage procapsids. The movement of a hinge is consistent with the density shifts observed by electron microscopy and in reconstructed images of procapsids and phage of P22, lambda, and T4 (Casjens, 1979; Prasad et al., 1993; Wurtz et al., 1976; Dokland and Murialdo, 1993; Kistler et al., 1976). The T4 coat protein undergoes partial refolding upon capsid expansion (Steven et al., 1990), as well as proteolytic cleavage. However, the expansion of both P22 and lambda

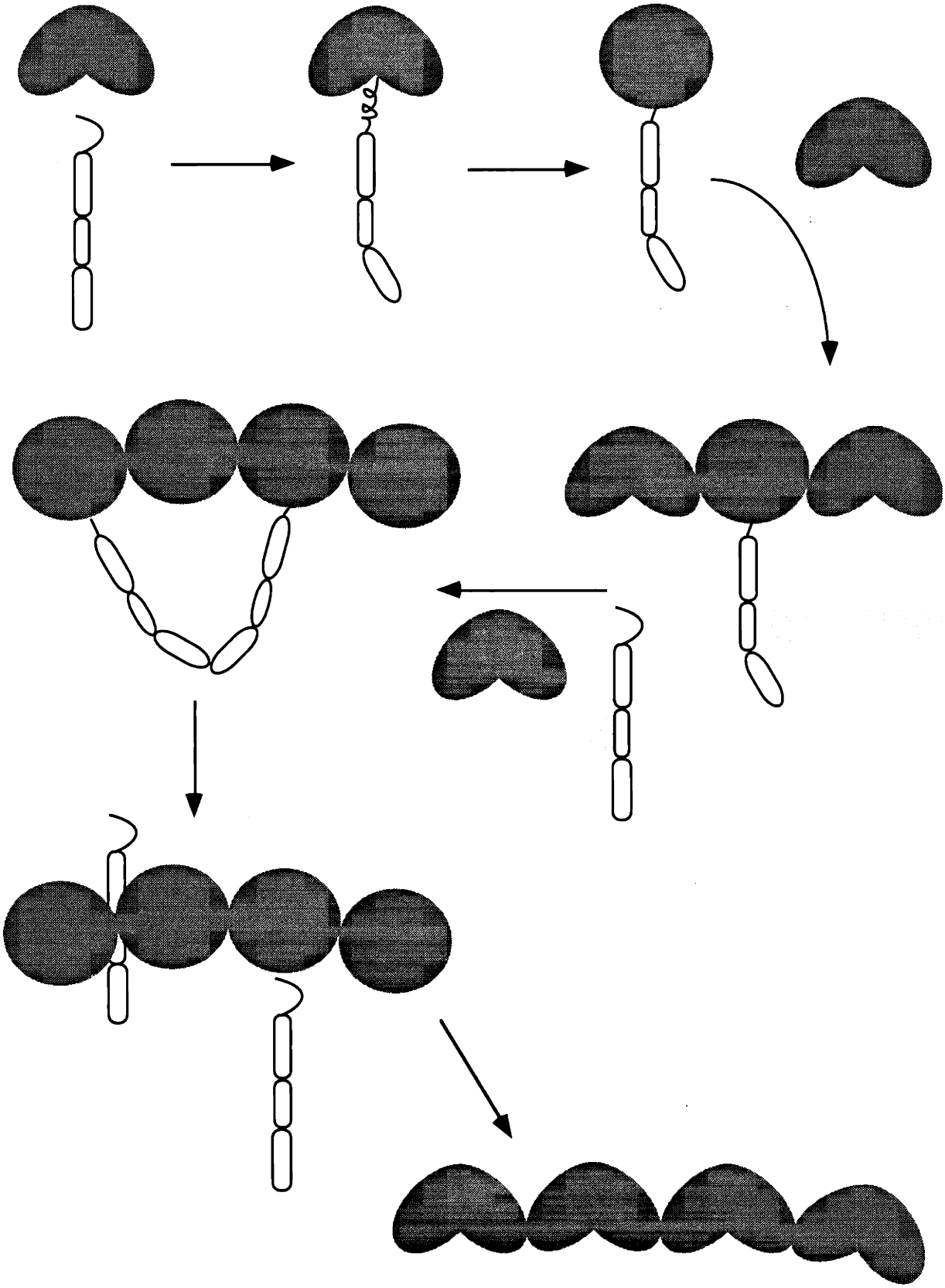


FIG. 5.2 A model for scaffolding/coat interactions during P22 procapsid assembly and maturation

procapsids to the mature form occurs with only very small secondary structure changes in the coat protein, (Prevelige et al., 1993a; Kawaguchi et al., 1983), implying that only relative movement of domains, as in a hinge opening, are involved.

Since there are no structures of monomeric coat protein subunits, it is not possible to compare conformational changes involved in the actual assembly pathway, but some conclusions can be drawn from the procapsid structure. In both P22 and lambda, the subunits at non-equivalent positions in the procapsid appear slightly different in shape (Prasad et al., 1993; Dokland and Murialdo, 1993, and see Fig. 5.3). These differences must involve the motion of large regions to be visible at the resolution of the image reconstructions. Dokland and Murialdo (1993) proposed that the differences could be caused by varied conformations of a hinge region. Alteration of a hinge may explain how the assembly of procapsids is inhibited by bis-ANS, a small hydrophobic dye molecule (Teschke et al, 1993). Bis-ANS also blocks the aberrant assembly of coat subunits in the absence of scaffolding, demonstrating that it must interact directly with the coat protein. The binding of bis-ANS did not alter the secondary structure of the coat protein, or its sensitivity to proteolytic cleavage (Teschke et al., 1993). Since bis-ANS binds to exposed hydrophobic patches in proteins, it might well be attached to the hinge site, locking it into the wrong conformation for assembly.

Does the scaffolding control the state of this hinge? Fuller (1979) had originally suggested the opposite, proposing that capsid expansion might cause scaffolding release by altering the scaffolding binding site. In light of the new cryo-EM structures of the P22 procapsid and phage, this mechanism seems less plausible, as expansion removes the holes that are thought to be

the scaffolding exit channels (Prasad et al., 1993). Scaffolding reentry experiments described in Chapter 2 confirmed that in contrast to empty procapsids, empty expanded capsids are not permeable to scaffolding subunits.

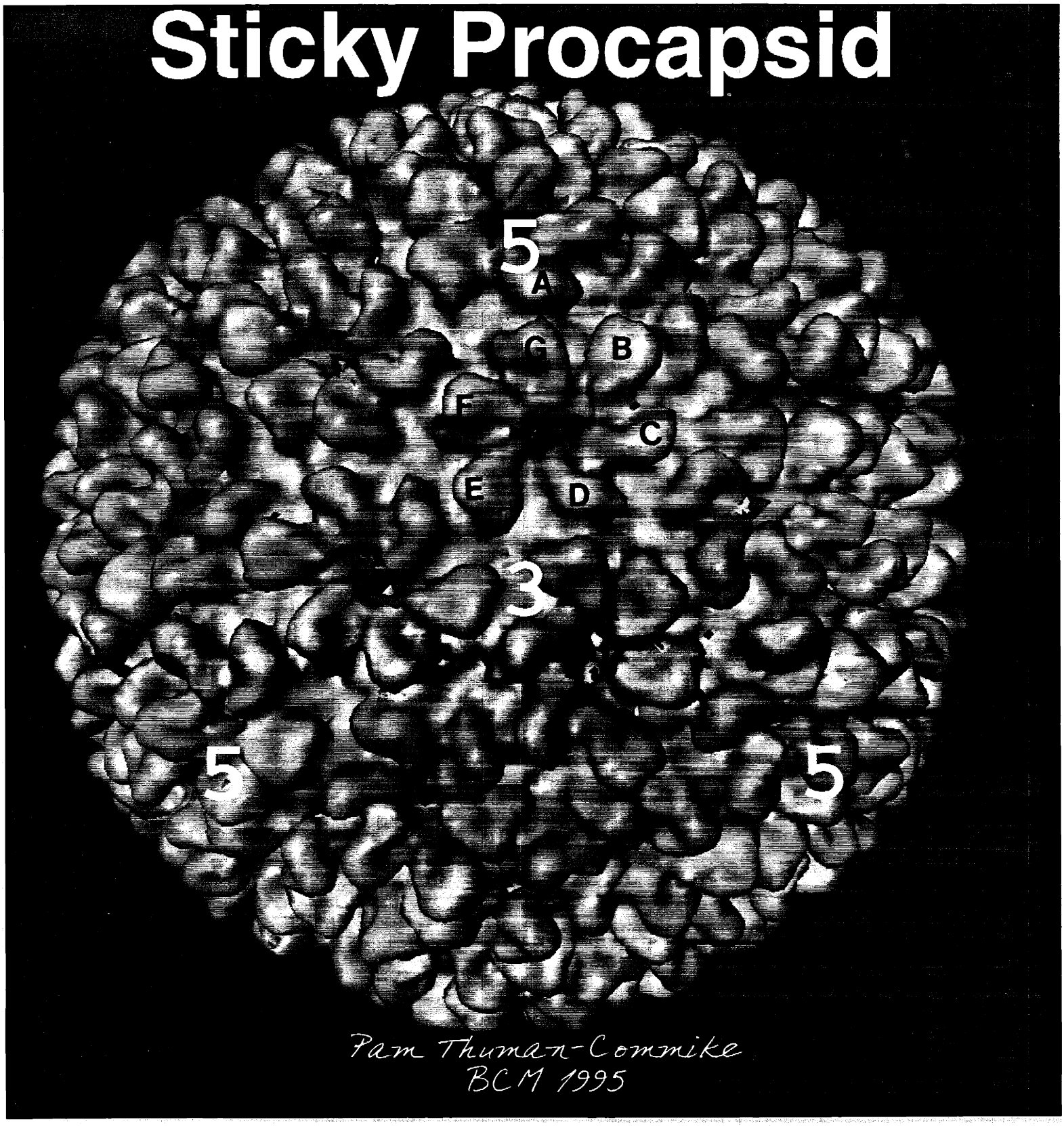
Several recent results suggest that scaffolding release is a prerequisite for capsid expansion, as predicted by my model, not the other way around. A careful analysis of the events that occur along the procapsid thermal denaturation pathway demonstrated that scaffolding release occurs at lower temperatures than capsid expansion (Galisteo and King, 1993). A mutant scaffolding (tsL177I) which is impaired in scaffolding release from procapsids also blocked capsid expansion, as discussed in Chapter 3. That scaffolding release is required for the hinge transition involved in expansion implies that scaffolding binding was required to establish the original procapsid conformation.

**Why do procapsids expand?**

All dsDNA phage undergo conformational changes during DNA packaging that result in capsid expansion and angularization of the capsid shape (King and Chiu, 1995). Adenoviruses may undergo similar transitions, judging from the distinct appearance of intermediate and mature particles in the electron microscope (D'Halluin et al., 1978a).

Reconstructed images of phage procapsids and mature virions reveal similar types of conformational changes in P22, lambda, and HK97 (Prasad et al., 1993; Dokland and Murialdo, 1993; Conway et al., 1995). The hexameric subunits of procapsids are arranged with a pronounced skewing towards the five-fold axes, so that the capsomeres have two-fold rather than six-fold symmetry (Fig. 5.3). The four subunits in the middle of the elongated ring

FIG. 5.3 Image reconstruction from cryo-electron micrographs of the P22 procapsid at 19 Å resolution (P. Thuman-Commike, B. Greene, J. Jakana. B.V. V. Prasad, P. Prevelige, J. King, and W. Chiu, manuscript in preparation). The procapsids used for this reconstruction were produced from cells infected with the scaffolding mutant strain L177I, originally thought to result in a "sticky" scaffolding protein, hence the title of "sticky procapsid." Numbers indicate the 5-fold and 3-fold symmetry axes. The letters identify the seven non-equivalent capsomeres of one asymmetric unit.


Sticky Procapsid
5
A
G
B
F
C
E
D
3
5
5
Pam Thuman-Commike
BCM 1995

(positions C, D, F, and G) are different from those at the ends. A similar skewed pattern is seen in the DNA-containing capsid of cauliflower mosaic virus (Cheng et al., 1992), suggesting that this arrangement might be a general mechanism necessary to build a T=7 shell. The hexamers of the mature phage capsids are much more regular, with clear six-fold symmetry. The mature form is more stable than the precursor (Steven et al., 1992), and the transition from procapsid to mature capsid has negative enthalpy, as demonstrated by calorimetry for P22 and T4 (Galisteo and King, 1993; Ross et al., 1985).

There must be some motivation for making a procapsid rather than the more energetically favored mature state. It is possible that the mature state cannot be directly reached from monomers, but must go through an intermediate. In the crystal structures of many viruses the subunits are seen to be held together by an intricate network of interlocking arms (Harrison, 1984; Liddington et al., 1991). The formation of these interactions probably requires that the subunits approach each other at particular angles. The procapsid lattice may provide the most favorable orientations for making these interactions, while transition to the mature state locks them into place after they have been made. The skewness of the procapsid may aid assembly by accentuating the differences between subunits in non-equivalent positions and allowing the required alternate types of contacts to be made.

Expansion was also proposed to have a function in DNA packaging (King and Chiu, 1995). While the structure of DNA within these viruses is not known, its insertion probably involves an initial interaction with the inner coat surface. The DNA cannot remain bound, however, since it must be ejected into the host cell. The expansion may serve to eliminate the DNA binding sites after a given amount of DNA has entered the capsid. In support of this idea, when DNA within a P22 capsid is destabilized by mutation of one

of the proteins that seals the portal, empty expanded capsids accumulate (Strauss and King, 1984). These capsids are not capable of packaging DNA (Poteete et al.,1979). The scaffolding may help to prevent premature expansion of the procapsid before the DNA has begun to enter. In those phage for which the substrate for DNA packaging is an already empty procapsid the role of expansion may be less critical – in contrast to P22, T4 can package DNA into expanded heads (Rao and Black, 1985).

It is worth noting that while expansion can be induced *in vitro* by heat or treatment with detergents (Galisteo and King, 1993; Ross et al., 1985; Wurtz et al., 1976), the back reaction, from expanded to procapsid state, has never been observed. The inclusion of an irreversible step may help to drive the assembly process foward.

**Regulation of lattice conformational transitions in other systems**

Large scale conformational transitions are a common feature in other viral life cycles. The picornaviruses are a family of small RNA-containing viruses, which includes both the polioviruses and the rhinoviruses (the cause of the common cold). The binding of these viruses to their receptors induces conformational changes that result in uncoating and RNA release (Rueckert, 1991). These conformational change can also be induced *in vitro* by treatment with heat or UV light (Roizman, 1959; Katagiri et al., 1967).

Characterization of numerous poliovirus mutants with a range of phenotypes has identified a small set of regions that regulate viral stability and ability to undergo confromational transitions (Chow et al., 1995). Interestingly, various single substitutions can lead to defects in both assembly and cell entry (Compton et al., 1990, Moscufo and Chow, 1992; Moscufo et al., 1993). One such mutant produces virions that are more labile to the

uncoating transformation, and also results in accumulation of many pentameric assembly intermediates that cannot proceed to form virions (Moscufo and Chow, 1992). The non-assembly competent dimers and trimers produced by temperature-sensitive P22 coat proteins (Teschke and King, 1995) may represent the same class of defect. These results reinforce the idea presented above that changes required for assembly may be the inverse of those required for later transitions.

These conformational changes in poliovirus can also be regulated by the binding of external factors. A class of antiviral drugs can block the uncoating step (McSharry et al., 1979), and also stabilize the virus against the heat-induced transitions *in vitro* (Caliguiri et al., 1980; Rombaut et al., 1991). The binding of several of these compounds has been studied by x-ray crystallography (Grant et al., 1994). All bind in a hydrophobic pocket on the inner viral surface. This site is normally filled with small lipid molecules called pocket factors which appear to stabilize the virus (Filman et al., 1989; Mosser and Rueckert, 1993). Binding of the drugs does not induce any obvious structural differences in the capsid, supporting a model in which the presence of the drug in the pocket blocks conformational changes that are required for uncoating (Grant et al., 1994). Presumably the normal pocket factors, which appear to be cell-associated (Mosser and Rueckert, 1993), must be removed before uncoating occurs. This is similar to the way in which mutant scaffolding proteins can block capsid expansion. Perhaps scaffoldings can be considered as a form of pocket factor.

The use of assembly or associated proteins to regulate conformational transitions involved in the growth of large protein polymers is a common theme in biology. Both tubulin and actin possess a host of associated or

binding proteins that control the nucleation, growth, and disassembly of microtubules or actin filaments (Hirokawa, 1994; Mandelkow and Mandelkow, 1995; Vandekerckhove, 1990; Kabsch and Vandekerckhove, 1992). The assembly of clathrin cages is assisted by assembly proteins which lower the critical concentration of clathrin needed to polymerize and regulate the size of the cages formed (Keen, 1990; Pearse and Robinson, 1990). These proteins add an extra level of regulation to the system, allowing the growth of the structure to be adjusted without modifying the structural protein of which it is composed.

These structures are inherently metastable as their function in the cell requires their assembly to be reversible. The assembled polymers may also have alternative forms; recent evidence suggests that the actin filament can exist in more than one distinct conformational state (Orlova and Egelman, 1993), and that the binding of regulatory proteins can control conformational switching of the polymerized lattice (Schmid et al., 1994). Thus the principles derived from examination of scaffolding proteins from simple phage systems may be applicable to far more complex cellular mechanisms.

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