Interactions between Coat and Scaffolding Proteins of Phage P22 Are Altered *in Vitro* by Amino Acid Substitutions in Coat Protein That Cause a Cold-Sensitive Phenotype†

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ABSTRACT: Cold-sensitive mutations in phage P22 coat protein cause the accumulation of precursor capsids in cells growing at the nonpermissive temperature (16 °C). The assembly of coat proteins which carry the substitutions threonine at position 10 to isoluecine (T10I), arginine at position 101 to cysteine (R101C), or asparagine at position 414 to serine (N414S) which cause cold-sensitivity has been investigated. All three proteins were found to fold into a monomeric species. Coat proteins carrying the amino acid substitutions T10I and R101C were not able to interact with scaffolding protein appropriately to initiate assembly *in vitro* while coat protein carrying the substitution N414S was able to assemble; however, capsids formed of this protein had an increased affinity for scaffolding protein. These amino acid substitutions define two regions in coat protein that are essential for the interaction of coat protein with scaffolding protein at different stages in capsid maturation.

The amino acid sequence of a multisubunit protein directs both its folding and its assembly process (Jaenicke, 1991; Seckler & Jaenicke, 1992; Teschke & King, 1992; Garel, 1992). Assembly of a multimer cannot begin until the folding of the monomeric subunit is sufficiently progressed so that the sites involved in the protein−protein interactions have formed (Garel, 1992). A viral capsid is an exemplary model of a multisubunit complex where hundreds of proteins must interact with each other in a specific sequence and with the appropriate proteins in order to form a complete capsid.

The initial product of assembly of the double-stranded DNA (dsDNA) containing bacteriophage, herpesviruses, and adenoviruses is a precursor capsid into which the dsDNA is actively packaged (Edvardsson *et al*., 1976; D'Halluin *et al*., 1978; Casjens & Hendrix, 1988; Lee *et al*., 1988; Sherman & Bachenheimer, 1988). This precursor capsid, the procapsid, of phage P22 is produced when 420 molecules of the 430 amino acid coat protein, product of gene 5, interact with 150−300 molecules of scaffolding protein encoded by gene 8. The minor phage proteins, the portal protein complex and pilot proteins, are also incorporated during the assembly. Scaffolding protein is necessary both for the correct assembly of coat protein and for the incorporation of the minor proteins (Earnshaw & King, 1978; Bazinet & King, 1988; Greene & King, 1996). To form the $T = 7$ icosahedral procapsid lattice, coat protein must assume four quasi-equivalent conformations: three conformations for the capsid hexamers which have a local 2-fold symmetry and an additional one for the capsid pentamers (Prasad *et al*., 1993; Thuman-Commike *et al*., 1996).

The mature phage is formed when DNA is actively packaged into the procapsid while scaffolding protein exits through holes in the procapsid lattice to be used in further rounds of assembly (King *et al*., 1973; King & Casjens, 1974). Exit of scaffolding protein must occur for maturation of the capsid to take place (Galisteo & King, 1993; Greene & King, 1996). Expansion of the capsid by 50 Å also occurs during packaging by the unbending of a hinge that causes a domain movement of coat protein (Earnshaw *et al*., 1976; Casjens, 1979; Prasad *et al*., 1993). Thus, not only must coat protein interact with other molecules of coat protein and scaffolding protein during assembly to adopt the correct positionally-dependent conformations, but it must also alter those conformations during DNA packaging.

Our approach to determine how the amino acid sequence directs both the folding and assembly pathways of coat protein is to select conditional lethal mutants that are affected in one or both of these processes. For instance, 18 temperature-sensitive mutants in coat protein form inclusion bodies at their nonpermissive temperature (Gordon & King, 1993). Five of the purified mutant proteins have been shown to fold into assembly-incompetent dimers and trimers *in vitro*, confirming that they were temperature-sensitive for folding (*tsf*)[1] mutants (Teschke & King, 1995; Galisteo *et al*., 1995). *In vivo*, the *tsf* coat protein mutants are rescued by overproduction of GroEL and GroES, implicating the chaperones in the folding of *tsf* coat proteins (Gordon *et al*., 1994).

Three cold-sensitive (*cs*) mutants of coat protein, T10I, R101C, and N414S, have a different phenotype than the coat protein *tsf* mutants. At the nonpermissive temperature (16 °C), the *cs* mutants assemble into procapsids but do not package DNA (Gordon, 1993; Greene and King, unpublished results). The *cs* procapsids produced at 16 °C contained portal protein at the concentrations usually seen when WT procapsids are isolated; however, T10I and R101C had

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[1] Abbreviations: WT, wild-type; *tsf*, temperature-sensitive for folding; *cs*, cold-sensitive; βME, β-mercaptoethanol; GuHCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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substantially lower concentrations of pilot proteins. Electron microscopy indicated that the procapsids produced appeared to be morphologically normal; that is, they were correct in shape and diameter. Nonetheless, closer examination of the mutant procapsids formed from T10I and R101C showed that they had lost their scaffolding protein, while procapsids made of N414S retained their scaffolding protein (Gordon, 1993; Greene and King, unpublished results). These data suggest that the cold-sensitive mutants might affect later stages in morphogenesis rather than folding. In this paper, we have investigated the effects of the *cs* amino acid substitutions on assembly *in vitro* and on the structure of the capsid lattice.

MATERIALS AND METHODS

Chemicals. Ultrapure GuHCl and urea were purchased from Schwarz/Mann ICN. Cyanogen bromide-activated Sepharose was from Pharmacia. HGT Seakem agarose was purchased from American Bioanalytical. All other chemicals were reagent grade and purchased from common sources.

Buffer. The buffer used in all studies was 50 mM Tris base, 25 mM NaCl, 2 mM EDTA, pH 7.6 with HCl. In refolding experiments, 0.2% βME was added to the buffer or 6 M GuHCl just prior to use.

Purification of cs Coat Proteins. Procapsids were prepared as previously described (Prevelige *et al*., 1988; Teschke & King, 1993; Galisteo *et al*., 1995). Briefly, *Salmonella typhimurium* growing at permissive temperature were infected with bacteriophage P22 carrying the cold-sensitive mutation in gene 5 in addition to the standard amber mutations that prevent phage maturation and cell lysis which cause the infected cells to accumulate procapsids. After 4 h of infection, the cells were concentrated by centrifugation, suspended in buffer, and frozen. The cells were lysed after one freeze/thaw cycle. The lysate was treated with RNase and DNase, and the procapsids were spun from the lysate at 80 000 rpm in a RP80AT rotor in a Sorvall RC M120EX centrifuge. The pelleted procapsids were suspended by incubating them in buffer on a Nutator (Clay Adams, Inc.) at 4 °C. After running the procapsids over a Sephacryl 1000 column, the scaffolding protein was extracted by incubating with 0.5 M GuHCl on ice and separated from empty procapsid shells by centrifugation as described above. T10I and R101C were extracted with GuHCl 1 time and N414S 3 times to remove the scaffolding protein. The proteins were stored in buffer at 4 °C as empty procapsid shells at 4 mg/mL.

Folding of Coat Protein Subunits. To dissociate the empty procapsid shells and unfold coat protein, the shells were diluted with 6 M GuHCl so that the final protein concentration was 1 mg/mL and the final GuHCl concentration was 4.5 M and incubated at room temperature for at least 30 min. Refolding by dialysis of the denaturant was done either for several hours at 4 °C with two changes of buffer or using a microdialyzer (Gibco-BRL Life Technologies) for 4−5 h with constantly exchanging buffer at 20 °C. Aggregated protein was removed by centrifugation or filtration. The concentration of the refolded protein was determined by the absorbance at 280 nm. Both 7.5% nondenaturing and 10% SDS−polyacrylamide gels were run on each sample to confirm protein conformation and concentration (Teschke & King, 1995).

Assembly of Procapsids in Vitro. Assembly of procapsids was done as described in Prevelige *et al*. (1988, 1993) and Teschke *et al*. (1993). The coat proteins that had been refolded at 4 or 20 °C were adjusted to the same concentration, usually between 0.4 and 0.7 mg/mL. For an assembly reaction, 120 μL of coat protein was added to a cuvette thermostated at 20 °C in an SLM Aminco-Bowman 2 spectrofluorometer. The settings for light scattering experiments were as follows: excitation wavelength, 500 nm; excitation band-pass, 4 nm; emission wavelength, 500 nm; emission band-pass, 4 nm; and PMT voltage, 500 V. Scaffolding protein was added so that the final concentration was 1 mg/mL. Assembly was monitored by the increase in light scattering. For some experiments, the assembly reaction was run on an agarose gel on which capsids are retained (Serwer *et al*., 1978; Galisteo & King, 1993). The agarose gel procedure is described below.

Scaffolding Protein Column. Three milligrams of scaffolding protein in 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3, was added to 0.6 g of CNBr-activated Sepharose (Pharmacia) that had been prepared following the instructions of the manufacturer. The coupling efficiency was 98% after the scaffolding protein had been reacted with the activated matrix overnight at 4 °C. Residual activated groups were blocked with glycine. The column was washed as suggested with low-pH buffer and high-pH buffer. This process yielded 1 mL of scaffolding protein−Sepharose. WT and *cs* mutant coat proteins (100 μL) that had been dialyzed using a microdialyzer at 20 °C as described above were applied to the column at 0.2 mg/mL and eluted with buffer with βME, and 200 μL fractions were collected. The elution profile of each protein was determined by reading the fluorescence of each fraction to assay for the presence of coat protein, which has six tryptophans, using an SLM Aminco-Bowman 2 spectrofluorometer with the excitation wavelength set to 280 nm, the emission wavelength to 340 nm, the band-passes each set to 4 nm, and the PMT voltage to 740 V.

Scaffolding Protein Extraction. Equilibrium scaffolding extractions were done by incubating WT or N414S procapsids overnight at 50 μg/mL in increasing concentrations of buffered GuHCl at room temperature. Conversion of procapsids to empty procapsid shells by the extraction of the scaffolding protein was monitored by the decrease in light scattering. Light scattering was measured on an SLM Aminco-Bowman 2 Spectrofluorometer with excitation and emission wavelengths set to 500 nm, the bandwidths to 4 nm, and the photomultiplier voltage to 450 V. The fraction of scaffolding protein remaining was determined by the formula $X_{obs} - X_{extract}/X_n - X_{extract}$ where X_{obs} is the observed light scattering, $X_{extract}$ is the value of the fully extracted empty shells, and X_n is the light scattering of the procapsids.

The kinetics of extraction of scaffolding protein from procapsids were monitored by light scattering with the fluorometer settings as described above except that the PMT voltage was set to 500 V. WT or N414S procapsids were plunged into a cuvette containing buffered 0.5 M GuHCl, and the change in light scattering was monitored. The data were fit with a computer using the formula for a first-order decay with two exponents utilizing the program Kaleidagraph (Synergy Software). To determine the amount of scaffolding protein extracted, the empty procapsid shells were pelleted from solution by spinning in a RP100AT rotor at 100 000 rpm for 10 min in a Sorvall RC M120EX centrifuge.

Heat Expansion of Empty Procapsid Shells or Procapsids. Empty procapsid shells or procapsids at 1 mg/mL comprising WT or *cs* mutant coat proteins were heated at 65, 68, or 71 °C for up to 30 min. Aliquots were removed every 5 min to tubes held on ice containing agarose gel sample buffer (40 mM Tris base, 1 mM EDTA, 20% sucrose, pH 8.3, with acetic acid). About 6 μg of protein was loaded onto 1.2% Seakem HGT agarose gels made with the same buffer without sucrose and run at 50 V for 4−5 h at room temperature. The gels were stained with Coomassie blue.

Sucrose Gradient Centrifugation. WT or N414S empty procapsid shells or shells that had been treated for 20 min at 71 °C at a concentration of 1 mg/mL were applied to a 2 mL 5−20% sucrose gradient and run in a RP55S rotor in a Sorvall RC M120EX centrifuge for 35 min at 35 000 rpm at 20 °C. Linear sucrose gradients were made using a Gradient Master Model 106 (Biocomp Instruments). The gradients were fractionated into 100 μL fractions by hand from the top, and a sample from each fraction was run on an SDS−polyacrylamide gel. The Coomassie blue stained gels were quantified using a Molecular Dynamics Computing Densitometer.

Urea Denaturation of Empty Procapsid Shells. WT and *cs* coat protein empty procapsid shells were incubated at room temperature for 24 h in increasing concentrations of buffered urea. The final concentration of empty procapsid shells in each tube was 1 mg/mL. The protein solutions were run on agarose gels as described above, loading 6 μg into each well.

RESULTS

The amino acid substitutions of the three *cs* mutants were determined by DNA sequencing of the region of the gene identified as carrying the *cs* mutations by the use of mapping plasmids (Gordon, 1993; Greene and King, unpublished results; Casjens *et al*., 1991). T10I and N414S were backcrossed into a wild-type background after they were identified and so should carry no other mutations (Jarvik & Botstein, 1973, 1975) while R101C has a known additional mutation in the gene encoding scaffolding protein, L177I, that is phenotypically silent (Greene and King, unpublished results). Purification of the three *cs* coat mutants was done essentially as previously described with WT and *tsf* coat protein preparations using phage strains that were defective in packaging DNA and in cell lysis, and growing the infected cells at permissive temperature (Prevelige *et al*., 1988; Teschke & King, 1995; Galisteo & King, 1993). Scaffolding protein was released from the procapsids by repeated incubation with 0.5 M GuHCl, pelleting the empty procapsid shells, and suspension of the shells in buffer (Fuller & King, 1981). The resulting empty procapsid shells were the storage form of the *cs* mutant proteins. About 50% of T10I and R101C empty procapsid shells expanded in the course of extraction of scaffolding protein with GuHCl.

Refolding of cs Coat Proteins. WT coat protein has been successfully refolded into assembly-competent monomers from denaturant with high yield both by dialysis and by rapid dilution of denaturant (Prevelige *et al*., 1988; Teschke & King, 1993). *Tsf* mutant coat proteins were also refolded to soluble species with high efficiency, albeit to dimers and trimers that were incompetent for assembly (Teschke & King, 1995; Galisteo *et al*., 1995). The folding of the *cs* mutants from denaturant was investigated to determine if they refolded into monomeric or multimeric species.

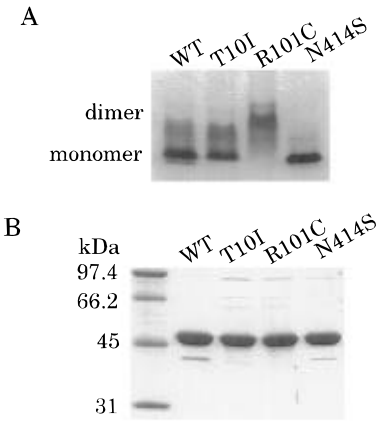

FIGURE 1: Nondenaturing (A) and SDS−polyacrylamide (B) gels of refolded WT and *cs* coat proteins. The coat proteins were refolded at 4 °C by dialysis, and the concentration of each was adjusted to 0.63 mg/mL. Each lane was loaded with 4 μg of refolded protein.

WT and *cs* shell stocks were incubated in 4.5 M GuHCl with 0.2% βME at 1 mg/mL to denature and dissociate the capsids. Refolding of the denatured proteins was accomplished by dialysis against two changes of buffer containing 0.2% βME at 4 °C. After diluting the refolded *cs* proteins to the same concentration, the proteins were run on a nondenaturing polyacrylamide gel (Figure 1A). A band with a mobility similar to WT coat protein monomers was observed in the samples of refolded T10I and N414S. Both WT and T10I displayed some dimers, while N414S had very little dimeric species. In this instance, R101C had a negligible amount of monomeric species with a predominance of species of higher oligomeric states; however, in other experiments where folding was done at 4 °C, R101C showed slightly higher quantities of monomeric species but at most 25% of the WT level. The reason for the variability in yield is unclear. In the lane of R101C, there was a broad smear between the bands for dimer and higher order oligomers suggesting an equilibrium between those species. An SDS−polyacrylamide gel was run on the refolded proteins to confirm that the same amount of protein had been loaded onto the native gel (Figure 1B). We have found that folding at higher temperatures leads to increased yields of monomeric T10I and R101C, suggesting that these mutants might be *cs* for folding (Fong and Teschke, manuscript submitted to *Biochemistry*).

Assembly of cs Coat Proteins. Procapsids can be assembled *in vitro* by mixing purified coat protein with purified scaffolding protein (Fuller & King, 1981; Prevelige *et al*., 1988). Assembly of procapsids is a nucleation-limited reaction in which coat protein has a critical concentration of 0.3 mg/mL below which assembly cannot happen (Prevelige *et al*., 1993). Assembly occurs via the addition of coat protein monomers to the growing edge of the capsid in a process that requires scaffolding protein since without scaffolding protein coat protein will polymerize in an uncontrolled fashion leading to the production of spirals (Earnshaw & King, 1978; Prevelige *et al*., 1990, 1993; Teschke *et al*., 1993; Teschke & King, 1995).

Assembly was initiated by the addition of scaffolding protein to WT and *cs* coat proteins incubated at 20 °C. The proteins in the experiment shown were folded by dialysis at

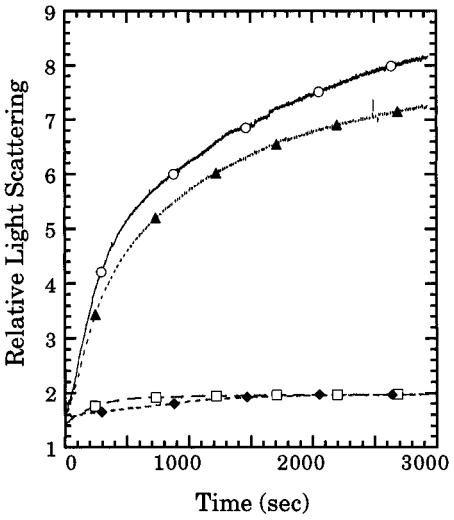

FIGURE 2: Assembly of refolded WT and *cs* coat proteins. Assembly was initiated by the addition of scaffolding protein to WT (○, solid line), T10I (□, long dashes), R101C (◆, short dashes), or N414S (▲, dotted line) held in a cuvette. The ability of the proteins to assemble was monitored by light scattering as described under Materials and Methods. The data were normalized so that the first point was equal to 1.

4 °C. Assembly of WT coat protein proceeded as expected (Figure 2) (Prevelige *et al*., 1988, 1993). N414S assembled with kinetics and yield that were similar to WT coat protein. T10I and R101C were assembly-incompetent, showing no assembly kinetics or yield of procapsids.

One possibility for the assembly-incompetence of T10I and R101C was that 20 °C was too low a temperature to permit assembly of these mutants or that folding at 4 °C leads to an assembly-incompetent conformation. Therefore, the proteins were refolded at 20 °C using a microdialyzer. Additionally, to test the effect of temperature on assembly, assembly reactions were done at temperatures from 20 to 28 °C and the assembly products run on an agarose gel to determine the yield of procapsids in each reaction (Figure 3). Increasing the temperature of the assembly reactions did not significantly increase the yield of procapsids for any of the proteins used. If T10I and R101C were affected at different steps in the assembly process, it was possible that mixing the refolded proteins together might allow assembly to occur; however, this was not the case. These data suggested that T10I and R101C are either inhibited in their ability to interact with scaffolding protein, perhaps in the same way or at the same step in assembly, or inhibited in the ability to interact appropriately with other coat protein subunits. T10I or R101C were assembly-incompetent whether the proteins were folded at 4 or 20 °C, suggesting that the temperature of folding was not the critical variable and that the inability to assemble must be due to a change in the folded conformation of the proteins.

Scaffolding and coat protein must interact during both nucleation and growth of the procapsid. The rate-limiting step in growth of the capsid is nucleation; once a nucleus is formed, then growth proceeds with such rapidity that it is hard to capture assembly intermediates (Prevelige *et al*., 1993). Consequently, it would be difficult to determine the effect of the mutant proteins on growth, but it was feasible to test the effect on nucleation (Prevelige *et al*., 1993). To investigate if the *cs* mutant coat proteins were inhibited in their ability to support nucleation, we mixed WT coat protein with T10I (Figure 4A) or R101C (Figure 4B) so that the total coat protein concentration was sufficient to support nucleation and growth (0.4 mg/mL), but the concentration of WT coat protein was so low that assembly was slow. If the presence of WT coat protein could help the mutant proteins form a nucleus, then we would have expected to observe kinetics close to those of WT coat protein at 0.4 mg/mL. However, the assembly kinetics of the mixed reactions were nearly the same as those of WT coat protein assembling at 0.133 mg/mL, which indicated that T10I and R101C were not able to nucleate even with the assistance of WT coat protein. Nevertheless, these data do not preclude the possibility that growth is also affected by the amino acid substitutions.

Prevelige and colleagues (Prevelige *et al*., 1993) did not observe assembly below 0.3 mg/mL coat protein. One difference between our protocols is that we dialyzed in buffer containing 0.2% βME. The single cysteine of coat protein is important for assembly *in vitro* since its modification with iodoacetamide inactivated WT coat protein without substantially altering its secondary structure (Eppler *et al*., 1991; P. Prevelige, personal communication). Perhaps the cysteine must be in the reduced form to be assembly-competent. Another difference between our protocols is that we monitor assembly with 90° light scattering in a fluorometer rather than by turbidity with a spectrophotometer. Light scattering is a more sensitive technique, so perhaps we can observe assembly at coat protein concentrations that Prevelige and colleagues could not. However, Prevelige and colleagues (Prevelige *et al*., 1993) determined the critical concentration

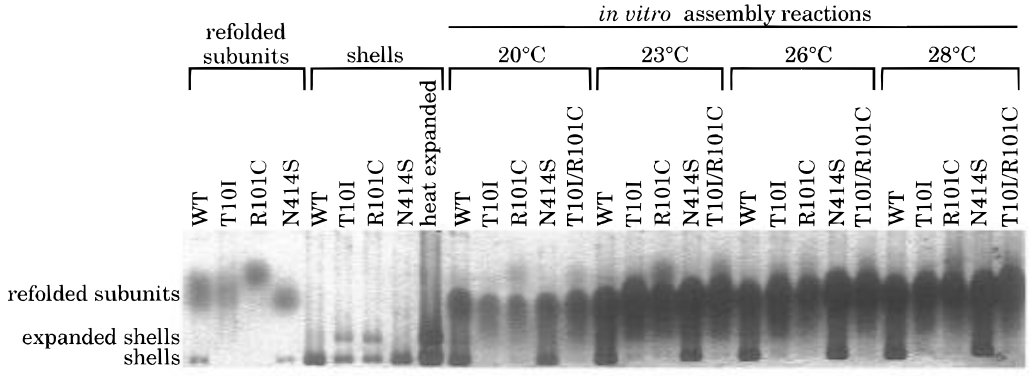

FIGURE 3: Assembly reactions of WT and *cs* coat proteins at different temperatures. Assembly reactions containing refolded WT or *cs* coat proteins and scaffolding protein were done overnight at the temperatures indicated. The reactions were run on a 1.2% agarose gel with each lane loaded so that it contained 2.7 μg of coat protein as described under Materials and Methods.

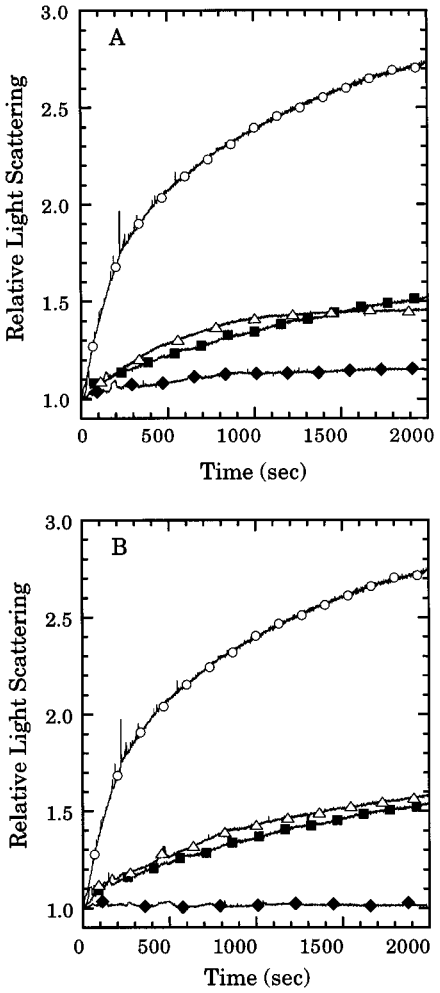

FIGURE 4: Assembly of WT coat protein mixed with *cs* coat proteins. Assembly reactions were initiated by the addition of scaffolding protein. The data were normalized so that the first point was equal to 1. Panel A shows the assembly of WT coat protein at 0.4 mg/mL (○), WT coat protein at 0.133 mg/mL (■), a reaction where 67% of the coat protein was T10I mixed with 33% WT coat protein at a final concentration of 0.4 mg/mL (△), and T10I at 0.267 mg/mL (◆). Panel B shows the assembly of WT coat protein at 0.4 mg/mL (○), WT coat protein at 0.133 mg/mL (■), a reaction where 67% of the coat protein was R101C mixed with 33% WT coat protein at a final 0.4 mg/mL (△), and R101C at 0.267 mg/mL (◆).

by several methods, so we believe the reason that we observe assembly at lower coat protein concentrations is unlikely to be due to instrumentation. Resolution of these differences must await further experimentation.

Interaction between Coat Protein and Scaffolding Protein. The assembly experiments did not address whether the assembly defect of T10I and R101C was due to an inability to interact with scaffolding protein or with other coat protein subunits. Additionally, the assembly experiments did not dismiss the possibility that N414S could have a change in interaction with scaffolding protein that was transparent during assembly. For instance, N414S could have an increased affinity for scaffolding protein which might not affect assembly kinetics but could affect a step later in phage biogenesis. To examine the interaction of the WT and *cs* coat proteins with scaffolding protein, the refolded proteins were applied to a column of scaffolding protein linked to Sepharose (Zopf & Ohlson, 1990). The concentration of coat proteins was held low so that insignificant assembly would

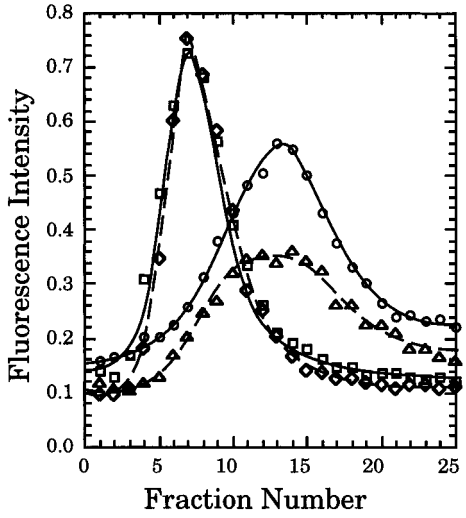

FIGURE 5: Elution of WT and *cs* coat proteins from a scaffolding protein−Sepharose column. The elution profiles of WT (○), T10I (◇), R101C (□), and N414S (△) were determined by tryptophan fluorescence.

occur while the proteins were passing through the column. The position of the protein eluted from the column was determined by monitoring the intrinsic fluorescence of the six coat protein tryptophans (Figure 5) (Eppler *et al.*, 1991; Teschke & King, 1993, 1995). The yield of eluted protein was 55−65% of the protein loaded onto the column. WT and N414S had the same elution volume, suggesting that they had similar affinities for scaffolding protein, in contrast to T10I and R101C which eluted substantially earlier than WT or N414S, indicating that they have decreased affinity for scaffolding protein. These data suggested that the *cs* effect of N414S must be exerted in another fashion than a change in its affinity for scaffolding protein during assembly and that T10I and R101C do not interact normally with scaffolding protein.

Interaction between the Coat Protein Lattice and Scaffolding Protein. A possible reason for the *cs* defect of N414S might be that the scaffolding protein is bound too tightly within the procapsid lattice, especially since scaffolding protein mutants have been identified which bind too tightly in the procapsid (Greene & King, 1996). Two classes of scaffolding protein have been identified within the procapsid lattice; about half of the scaffolding molecules appear to be bound specifically by the lattice while the other half apparently occupy spaces between the specific scaffolding molecules (Green & King, 1994). The extraction of scaffolding protein from procapsids shows two kinetic phases as does reentry of scaffolding into empty procapsid shells (Teschke *et al.*, 1993; Greene & King, 1994). If an increase in the affinity of scaffolding protein for the coat protein lattice was the reason for the *in vivo* phenotype of N414S, then DNA packaging might be aborted when scaffolding protein did not exit in response to docking of DNA at the portal complex.

To test whether scaffolding protein was bound more tightly within procapsids made of N414S than those of WT coat protein, scaffolding protein was extracted by overnight incubation in increasing concentrations of GuHCl. The amount of scaffolding protein remaining within the procapsids was measured by changes in light scattering, since procapsids scatter more light than empty procapsid shells (Greene & King, 1994). Changes in the concentration of

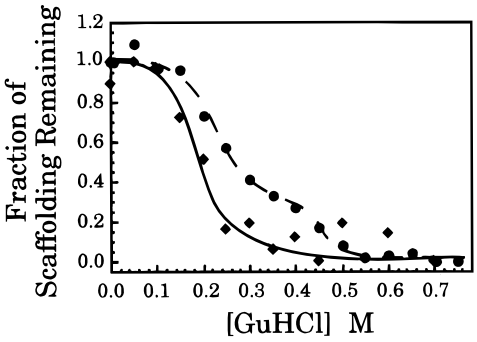

FIGURE 6: Extraction of scaffolding protein from WT (◆, solid line) or N414S (●, dashed line) procapsids by incubation with urea. The fraction of scaffolding remaining within procapsids at each urea concentration was determined by light scattering at 500 nm by the formula described under Materials and Methods. The lines are drawn to aid the eye and are not meant to represent the fit of the data to any model.

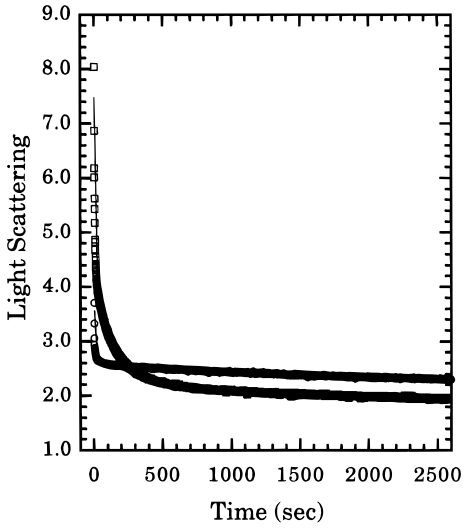

FIGURE 7: Kinetics of extraction of scaffolding protein from WT (○) or N414S (□) procapsids. The procapsids were plunged into a cuvette containing 0.5 M GuHCl, and the extraction was monitored by the change in light scattering due to the exit of scaffolding protein. The lines shown are the fit of the data to a first-order decay with two exponents.

GuHCl needed to extract 50% of the scaffolding protein would indicate a change in the binding affinity of scaffolding protein within the capsid lattice. In Figure 6, the scaffolding protein remaining within WT or N414S procapsids with increasing concentrations of GuHCl is shown. The curve of scaffolding extraction from WT procapsids is similar to that seen in Greene and King (1994) although the curve is shifted to lower GuHCl concentrations most likely due to the lower procapsid concentration used in our experiments. Nonetheless, clearly higher GuHCl concentrations were required to remove scaffolding protein from procapsids of N414S. Additionally, a plateau was seen in the N414S curve at higher GuHCl concentrations perhaps the result of changes in the binding of both classes of scaffolding protein within the capsid lattice of N414S. Greene and King (1996) have also observed a plateau in the extraction of one of their scaffolding protein mutants.

To determine if the activation energy required to extract scaffolding protein from within the capsid lattice had been altered, the kinetics of extraction were measured (Figure 7). WT or N414S procapsids were rapidly diluted into 0.5 M GuHCl, a concentration sufficient to extract scaffolding protein from WT and N414S procapsids but not enough to denature the lattice (Prevelige *et al*., 1988; Teschke & King, 1993; Greene & King, 1994, Figure 6). The decrease in light scattering seen when either WT or N414S was plunged into 0.5 M GuHCl was fit with the formula for a first-order decay with two exponents. The fast phase of the extraction of scaffolding protein from WT procapsids had a relaxation time of 4 s and that from N414S had a relaxation time of 6 s. These data indicated that the activation energy required to disrupt the scaffolding protein−scaffolding protein interactions that hold the "loose" scaffolding protein within the procapsid was essentially unaffected by the amino acid substitution in the procapsid lattice. The slow kinetic component of the scaffolding exit from WT procapsids had a relaxation time of 1594 s whereas the exit from N414S procapsids had a relaxation time of 210 s, suggesting that the activation energy required for the release of the "tight" scaffolding protein was decreased by the amino acid substitution in coat protein. To verify that the decrease in light scattering was due to exit of scaffolding protein from the procapsids, the extractions at 0.5 M GuHCl were centrifuged with conditions set so that the capsid structures would pellet. The protein in the supernatant and pellet was analyzed by SDS−PAGE. In both cases, the supernatant contained nearly all and equal amounts of the scaffolding protein initially present in the each procapsid species, indicating that efficient extraction had occurred (data not shown).

In both the equilibrium and kinetic extraction experiments, the initial light scattering of the N414S procapsids was reproducibly about 2-fold higher than WT procapsids even though the protein concentrations were adjusted to be the same. Additionally, the composition of the protein within WT or N414S capsids was nearly identical, and differences between them were not enough to be responsible for this drastic change in light scattering (data not shown). This observation, along with the results discussed above, can be rationalized if the scaffolding protein within procapsids of N414S is bound differently than within WT procapsids.

Changes in the Structure of the Capsid Lattice of cs Coat Proteins. All three *cs* coat protein mutants have changes in their ability to bind scaffolding protein once polymerized into the procapsid; T10I and R101C were unable to retain scaffolding protein, and N414S displayed increased affinity for scaffolding protein. Since the scaffolding protein is wild-type in each case, these changes in affinity must be due to a change in the coat protein lattice of each mutant. To investigate changes in the capsids of the *cs* mutants, heat expansion and urea denaturation experiments were done.

Expansion of procapsids to a lattice with many of the characteristics of mature phage capsids can be induced by heat (Galisteo & King, 1993). Expansion occurs during DNA packaging, closing the holes in the procapsid to protect the DNA within. During expansion, there is a 50 Å increase in the diameter of the capsid which can be easily observed on an agarose gel (Serwer *et al*., 1986; Galisteo & King, 1993). Empty procapsid shells were heated at 65, 68, or 71 °C. At the times indicated in Figure 8, aliquots were moved to ice. The samples were run on an agarose gel designed to resolve procapsids from expanded capsids.

At 65 °C, WT empty procapsid shells (band 1) began to expand within 5 min of incubation (band 2). The portion of expanded shells increased with time, but even after 30

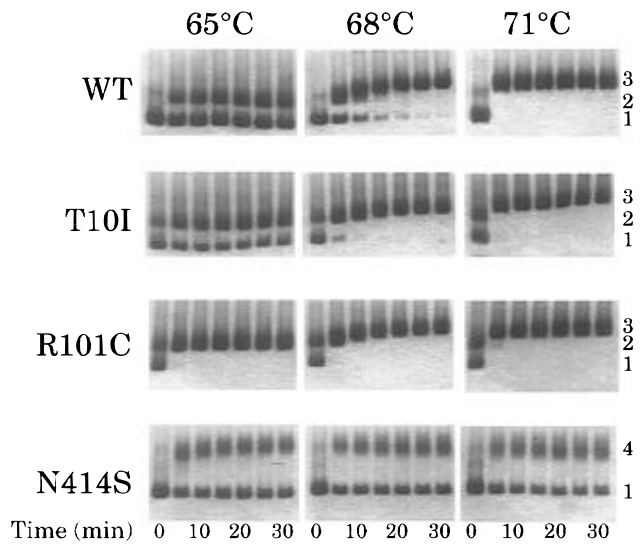

FIGURE 8: Heat expansion of WT and *cs* empty procapsid shells. Shells were incubated at the temperatures indicated at 1 mg/mL. Every 5 min, an aliquot was moved to ice. The samples were run on 1.2% HGT agarose gels as described under Materials and Methods. The species that occur during expansion are indicated as follows: (1) corresponds to empty procapsid shells, (2) expanded shells, (3) expanded shells with an unfolded domain, and (4) the slowest migrating species seen only in the expansion of N414S.

min, only approximately 50% of the empty procapsid shells had expanded. The kinetics of expansion of T10I were similar to those of WT shells at this temperature. R101C had completely different expansion kinetics; even at 5 min, nearly all of the shells had expanded. Interestingly, N414S expanded to a species that ran more slowly on the agarose gel (band 4). This expansion was rapid, occurring within 5 min and with about 50% of the shells expanded to this form. There was a decrease in the mobility with the time of incubation, most likely indicating that the species is increasing in diameter. This species has a mobility less than the slowest migrating species identified by Galisteo and King (band 3) (1993). These data suggested that there were two populations within the N414S sample: one that expanded at an abnormally low temperature to a form different than WT expanded capsids and another that is unable to expand at all.

At 68 °C, WT empty procapsid shells (band 1) expanded to the species that is similar to mature phage capsids (band 2). With increasing time at 68 °C, a further expansion to a species identified by Galisteo and King (1993) as an expanded capsid with an unfolded domain was observed (band 3). At 30 min, only a small amount of unexpanded shells remained. T10I was also converted to the expanded form with an unfolded domain (band 3) with kinetics that were substantially faster than WT: by 10 min, nearly all of the shells had been converted to expanded forms. R101C was completely converted to expanded forms within the first 5 min. A portion of N414S again expanded to the slowest migrating species (band 4) with the same fraction expanding as did at 65 °C. The data from expansion at 65 and 68 °C indicated that the coat protein lattice of T10I and R101C had been altered by these amino acid substitutions in a way that causes them to respond more readily to heat than WT shells. Within 5 min at 71 °C, WT, T10I, and R101C had expanded to the species with an unfolded domain (band 3). N414S was unchanged in comparison to 68 or 65 °C. When procapsids, rather than empty procapsid shells, made of WT or N414S coat protein were heat-expanded, the same species were present, indicating that the absence of scaffolding protein from the shells did not affect the expansion (data not shown). This was the expected result since scaffolding protein exits from WT procapsids at 44 °C, well below the temperature needed to cause expansion (Galisteo & King, 1993).

We postulated three explanations for the aberrantly expanded form of N414S. The first hypothesis was that the expanded forms were particles that had increased in diameter. The second notion was that the expanded form was not actually of different diameter but that during expansion a surface charge was exposed or concealed that would alter the charge to mass ratio, which in turn would decrease its mobility in the agarose gel. Third, the N414S capsids could have dissociated in response to heat into discrete small oligomers such as pentamers or hexamers that migrated to the position seen on the agarose gel. Sucrose gradient sedimentation was done on samples of WT and N414S empty procapsid shells that were heated or untreated to determine if any of these conjectures were valid (Figure 9). WT empty procapsid shells migrated to a peak centered at fractions 11−13 whereas WT shells heated at 71 °C for 20 min to induce expansion migrated to fractions 8−9. The expanded capsids had a reduced migration in the gradient because the increase in surface area without a change in protein mass leads to an increase in the frictional coefficient (Galisteo & King, 1993). Empty procapsid shells of N414S migrated to a peak with a center at fractions 10−13, a similar migration as seen in the gradient of WT shells. After treatment at 71 °C for 20 min, there were two peaks seen: one centered at fractions 11−12 corresponding to empty procapsid shells and another at fractions 7−8. A peak which contains particles with a slower migration in a sucrose gradient was consistent with either a capsid that had a larger surface area to mass ratio or smaller particles such as pentamers or hexamers that would migrate less far in the gradient. To distinguish between these possibilities, the light scattering at 500 nm of fractions 7 and 12 was determined. If the particles in the fractions migrating toward the top of the gradient were pentamers or hexamers, the light scattering should decrease; however, the light scattering of fraction 7 was approximately the same as the light scattering in fraction 13 when normalized for the amount of protein in each. These data were consistent with the slow migrating peak containing shell structures. To confirm that small oligomers were not present in the heated N414S sample, a 6% nondenaturing acrylamide gel was run on heated and nonheated WT and N414S empty procapsid shells. No species were observed to migrate into the resolving gel, indicating that there were no small oligomers present (data not shown).

The heat expansion data suggested that the conformation of each *cs* coat protein in the procapsid lattice had been altered from that of WT. To test whether the stability of the lattice had been affected, we did urea denaturation studies. Empty procapsid shells were incubated overnight with increasing concentrations of urea. Each sample was applied to an agarose gel to determine at what urea concentration the transition from shells to denatured coat protein monomers occurred (Figure 10).

WT empty procapsid shells (band 1) were stable to concentrations of urea up to 5.25 M with the transition to

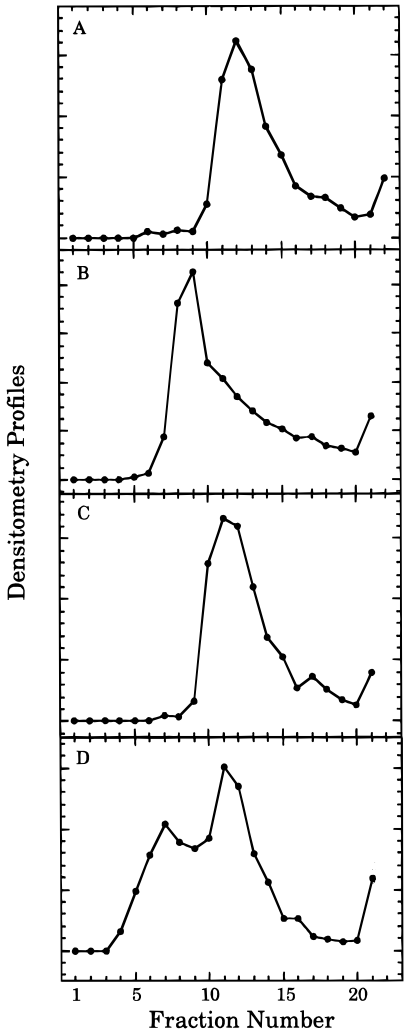

FIGURE 9: Sucrose gradient sedimentation of WT and N414S empty procapsid shells and expanded shells. Shells composed of WT or N414S were incubated at 71 °C for 20 min or held on ice. The panels are (A) WT shells, (B) heated WT shells, (C) N414S shells, and (D) heated N414S shells. Samples of each were applied to a 5−20% sucrose gradient and centrifuged as described under Materials and Methods. The gradients were fractionated from the top into 21−22 100 μL fractions. The protein content of each fraction was analyzed by SDS−polyacrylamide gels and densitometry.

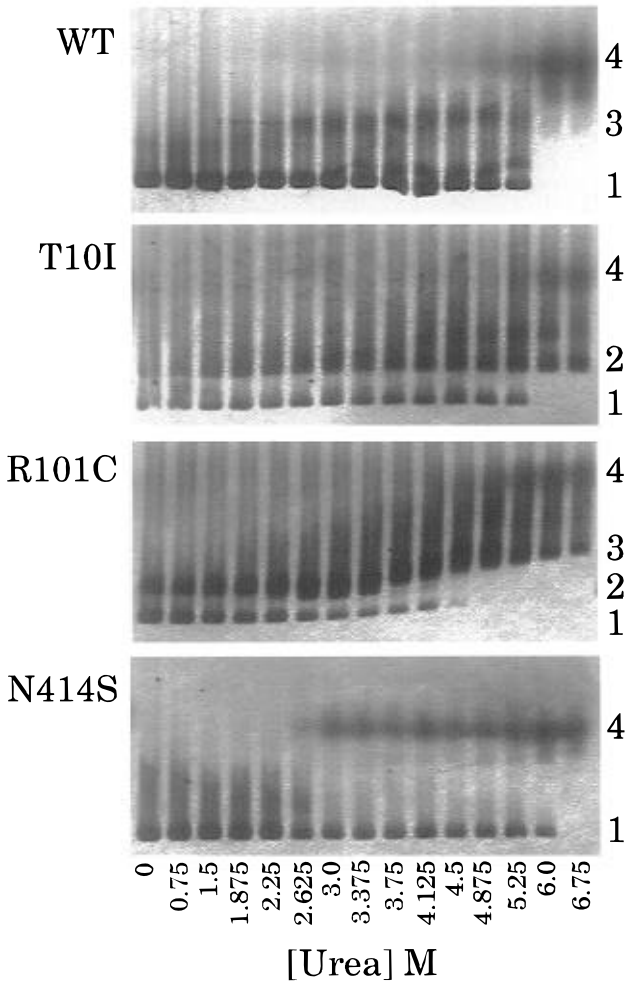

FIGURE 10: Urea denaturation of WT and *cs* empty procapsid shells. Shells at a final concentration of 1 mg/mL were incubated in increasing concentrations of urea overnight as indicated on the bottom of the figure, and samples were run on 1.2% HGT−agarose gels. The species that occur at the various urea concentrations are indicated on the right side of each panel as follows: (1) empty procapsid shells; (2) expanded shells; (3) expanded shells with an unfolded domain; and (4) monomeric coat protein.

denatured coat protein monomers (band 4) occurring by 6 M urea. These data closely correlated with the denaturation of WT shells previously analyzed by fluorescence and light scattering (Foguel *et al.*, 1995). As the urea concentration increased, a small amount of a species postulated by Galisteo and King (1993) to be expanded shells with the unfolded domain (band 3) was present. The analysis of the denaturation of T10I is complicated by the mixed population of empty procapsid shells (band 1) and expanded shells (band 2) that were present in our stocks; however, the denaturation of the empty procapsid shells of T10I had a transition that was similar to WT shells which suggested a similar stability. The empty procapsid shells of R101C expanded (band 2) in response to the urea, with the majority of the shells expanding by 4.125 M urea. The expanded shells then underwent a further transition to expanded capsids with an unfolded domain (band 3). This transition occurred from 3.75 to 6.0 M urea. These expanded capsid species were denatured by higher urea concentrations (data not shown). Since R101C empty procapsid shells undergo expansion prior to denaturation, it was difficult to definitively determine their stability. Empty procapsid shells of N414S (band 1) displayed an extremely broad transition with denaturation to monomers (band 4) occurring between 2.625 and 6.0 M urea with no expansion apparent during denaturation. One possibility for the broad transition is that the shells of N414S were substantially destabilized as compared to WT shells, thereby implicating this asparagine in intersubunit interactions. A more likely explanation is that there were two populations in the N414S empty procapsid shells: one that denatured with a sharp transition between 2.625 and 3.0 M urea and another population with a sharp transition between 6.0 and 6.75 M urea. The latter explanation is consistent with all of the observations of the behavior of the procapsid lattice of N414S and again implicates this amino acid substitution in intersubunit interactions, sometimes causing a decrease in lattice stability and sometimes causing an increase in the stability of the lattices.

DISCUSSION

Coat protein passes through several conformations as it develops into a mature phage: nascent polypeptide chains,

folding intermediates, folded monomeric subunits, procapsids, and, finally, the mature bacteriophage. Throughout these processes, coat protein must interact with other proteins. During folding, coat protein may interact with molecular chaperones; *tsf* mutants in coat protein have been shown to interact with GroEL and GroES during folding (Gordon *et al.*, 1994; Nakonechny and Teschke, unpublished results). During assembly, coat protein must interact with scaffolding protein and other coat protein subunits to form a correctly dimensioned closed icosahedral lattice. Here we have shown that the amino acid sequence not only directs the folding pathway of phage P22 coat protein but also dictates the protein−protein interactions necessary for its assembly as well.

The three amino acid substitutions that cause a *cs* phenotype *in vivo* have two different effects on assembly *in vitro*. T10I and R101C fold into monomeric subunits but are not assembly-competent. They do not interact with scaffolding protein with the same affinity as WT coat protein and are not able to form an initiation complex even with the assistance of WT coat protein; growth may be inhibited as well. N414S also folds into monomers, but these are competent for assembly. Although all three *cs* proteins fold into monomers, clearly T10I and R101C have folded into a conformation that is different from WT coat protein. Differences in limited proteolysis patterns suggest that N414S may have an altered monomeric conformation as well (Fong and Teschke, manuscript submitted to *Biochemistry*).

It is perplexing that refolded T10I and R101C are not assembly-competent *in vitro* but are able to fold and assemble into procapsids *in vivo*. This conundrum can be resolved if there is a chaperone used in the folding of these *cs* proteins. While GroEL and GroES do not rescue phage production of the *cs* mutants at 16 °C, it is possible that another chaperone might assist in the folding (Fong and Teschke, manuscript submitted to *Biochemistry*). A most intriguing possibility would be the use of cold-induced chaperones in the folding of T10I and R101C (Lelivelt & Kawula, 1995). Another interesting possibility is that the portal protein complex (gp1) or pilot proteins (gp7, gp16, gp20) might be crucial for assembly *in vivo*. Portal protein is not essential for assembly of a WT capsid *in vivo* or *in vitro*; the kinetics of assembly are the same in the presence or absence of portal protein (Bazinet & King, 1988). However, it is likely that portal protein is somehow involved in initiation since only one portal complex is incorporated into a capsid. Perhaps the presence of portal protein is crucial to the formation of a procapsid for the *cs* coat protein mutants since all of the mutant procapsids have normal amounts of portal protein regardless of the growth temperature (Gordon, 1993; Greene and King, unpublished results). The pilot protein, gp16, has been shown to accelerate the kinetics of assembly of WT coat protein *in vitro*, and perhaps it is gp16 that is needed for proper assembly of the *cs* mutants (Thomas & Prevelige, 1991). Interestingly, N414S has a normal complement of gp16 even when grown at 16 °C while T10I and R101C have decreased amounts of gp16 when grown at nonpermissive temperatures. Greene and King (1996) have found several scaffolding protein mutants that fail to recruit gp16 into procapsids.

The procapsid lattices of the *cs* mutants are altered even when formed *in vivo* at permissive temperatures. The binding of scaffolding protein was diminished within the capsid lattice of T10I and R101C but was bound more tightly within the lattice composed of N414S. During maturation, the procapsid lattice must expand into the mature phage lattice. T10I and R101C expanded more readily than WT coat protein lattices while N414S had two populations: one that did not expand at all and another that expanded to a larger than normal form. Urea denaturation demonstrated alterations in the lattices of all three mutants.

Taken together, these data suggest that the structure of the N-terminal portion of coat protein is especially important in the formation of the assembly initiation complex with scaffolding protein. Both the N-terminus and the C-terminus must be involved in retention of scaffolding protein within the coat protein lattice. A recent 19 Å structure of the P22 procapsid done by image reconstruction of cryo-electron micrographs showed a region of coat protein density or "fingers" under the capsid hexamers that were suggested to interact with scaffolding protein (Thuman-Commike *et al.*, 1996). The putative location of the fingers is logical since scaffolding most likely exits through the hole in center of each hexamer (Prasad *et al.*, 1993). Perhaps the *cs* amino acid substitutions are involved in the formation of these "finger" structures.

Thuman-Commike and colleagues (Thuman-Commike *et al.*, 1996) suggest that scaffolding protein prevents the coat protein lattice from premature expansion and that exit of scaffolding protein allows the domain movement needed for closure of the holes. This notion fits well with the data gathered from experiments done using capsids of T10I and R101C. In these mutants, the scaffolding protein exited prematurely, and the capsids expanded in response to heat too readily. How does N414S conform with this idea? Conceivably, N414S subunits do not efficiently transmit the signal needed for expansion, but in the population where that signal is conveyed, the domain movement is too large as if the WT asparagine residue at position 414 is a doorstop and in its absence the hinge can swing the domain out too far. This idea would explain why N414S has two capsid populations. Regardless, the scaffolding protein within the procapsid lattice of N414S must be bound in an unusual fashion evidenced by the greater amount of GuHCl needed to extract the scaffolding protein, the increased rate of extraction, and the higher light scattering intensity. These data can be explained by the energy coordinate diagram shown in Figure 11. In this diagram, we have set the energy of the extracted scaffolding protein to be the same for each species of procapsid since the scaffolding protein is wild-type in both cases. The diagram shows that scaffolding protein within N414S procapsids is bound more tightly (larger ΔG) than within procapsids composed of WT coat protein because it has a lower energy in the bound state. The activation energy required for the transition state is diminished (decreased $\Delta G^{\ddagger}$) in the extraction of scaffolding proteins from within N414S procapsids, causing the kinetics of extraction to be faster. Elucidation of the differences in the interactions by which scaffolding protein is bound within the lattice composed of N414S coat protein will require further examination.

The information needed to fold and assemble coat protein is spread throughout its amino acid sequence. The three *cs* mutants have identified new roles for regions of coat protein involved in coat protein−scaffolding protein interactions, in coat protein−coat protein interactions, and in conformational

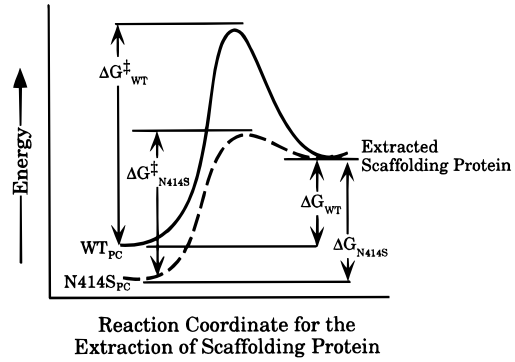

FIGURE 11: Reaction coordinate diagram for the extraction of scaffolding protein from within procapsids of WT (solid line) or N414S (dashed line) coat proteins. WT_{PC} and $N414S_{PC}$ represent WT scaffolding protein within procapsids composed of WT or N414S coat protein. The reaction coordinate was drawn based on data from the extraction of scaffolding protein shown in Figures 6 and 7.

transitions. The *tsf* amino acid substitutions have identified amino acids that are critical for the proper folding pathway of coat protein (Gordon & King, 1993; Teschke & King, 1995; Galisteo *et al*., 1995). We are continuing to analyze conditional lethal mutants as a nonspecific probe to determine the amino acids critical for the folding and assembly of coat protein.

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