The Folded Conformation of Phage P22 Coat Protein Is Affected by Amino Acid Substitutions That Lead to a Cold-Sensitive Phenotype†

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ABSTRACT: Three cold-sensitive mutants in phage P22 coat protein have been characterized to determine the effects of the amino acid substitutions that cause cold sensitivity on the folding pathway and the conformation of refolded coat protein. Here we find that the three cold-sensitive mutants which have the threonine residue at position 10 changed to isoleucine (T10I), the arginine residue at position 101 changed to cysteine (R101C), or the asparagine residue at position 414 changed to serine (N414S) were capable of folding from a denatured state into a soluble monomeric species, but in each case, the folded conformation was altered. Changes in the kinetics of folding were observed by both tryptophan and bisANS fluorescence. In contrast to the temperature-sensitive for folding coat protein mutants which can be rescued at nonpermissive temperatures *in vivo* by the overproduction of molecular chaperones GroEL and GroES [Gordon, C. L., Sather, S. K., Casjens, S., & King, J. (1994) *J. Biol. Chem. 269*, 27941−27951], the folding defects associated with the cold-sensitive amino acid substitutions were not recognized by GroEL and GroES.

Cold-sensitive mutations have traditionally been used to define and order genes that are involved in development in a variety of organisms, including *Drosophila*, yeast, viruses, and *Escherichia coli* (Rosenbluth *et al.*, 1972; Jarvik & Botstein, 1973, 1974; Singh & Manney, 1974; Somers & Kit, 1973; Guthrie *et al.*, 1969). Cold-sensitive mutations have been shown to affect the assembly of macromolecular structures such as ribosomes, tubulin spindles, and F-actin *in vivo* (Guthrie *et al.*, 1969; Hiroka *et al.*, 1984; Chen *et al.*, 1993). However, the mechanism by which an amino acid substitution leads to a cold-sensitive (cs)[1] phenotype is not well-understood.

The simplest explanation for a cold-sensitive phenotype is that hydrophobic interactions needed to stabilize multisubunit proteins are decreased by the amino acid substitution leading to subunit dissociation or improper assembly. Another explanation for cold sensitivity of a mutant protein is that the amino acid substitution causes a decrease in the overall hydrophobicity of the protein, making it more sensitive to the diminution of the hydrophobic effect at lower temperatures, thereby causing conformational changes or denaturation. However, cold denaturation or dissociation of proteins *in vitro* has been observed only under extreme conditions of temperature or pH or with the addition of denaturant (Privalov *et al.*, 1986; Chen & Schellman, 1989; Griko *et al.*, 1988; Griko & Privalov, 1992; Jaenicke, 1987; Lissin *et al.*, 1990). Since these conditions do not exist *in vivo*, it seems likely that another mechanism aside from a generalized decrease in hydrophobicity underlies cold sensitivity *in vivo*. One possible scenario for a cs phenotype is that a protein might need some structural flexibility to perform its function and that the cs amino acid substitution would lock the protein into a conformation such that the necessary flexibility in the polypeptide chain would be diminished at low temperatures. Another cause might be that folding into the appropriate conformation is cold-sensitive and the protein never achieves the correct and functional folded state at low temperatures (Han *et al.*, 1990; Mutero *et al.*, 1994). Here we have explored the effect of three amino acid substitutions in phage P22 coat protein that lead to a cold-sensitive phenotype.

Coat protein of phage P22 provides a model where the information content for both folding and assembly carried in the amino acid sequence can be probed independently since folding can be uncoupled from capsid assembly *in vitro*. Consequently, the effect of an amino acid substitution on each pathway can be determined (Prevelige *et al.*, 1988; Teschke & King, 1993, 1995). The icosahedral shell of P22, a double-stranded DNA bacteriophage of *Salmonella*, consists of 420 chemically identical coat protein subunits. Each coat protein subunit is a 47 kDa polypeptide of 430 amino acids (Botstein *et al.*, 1973; Eppler *et al.*, 1991). Following the synthesis and folding into assembly-competent subunits, coat protein interacts with 150−300 molecules of a 33 kDa scaffolding protein which is required for the polymerization reaction leading to the procapsid, a precursor capsid (King *et al.*, 1973; King & Casjens, 1974; Fuller & King, 1980; Prevelige *et al.*, 1988). Assembly of the procapsid proceeds by the addition of monomers of coat protein to the growing edge of the partially formed capsid (Prevelige *et al.*, 1993). Several copies each of three pilot proteins and a portal protein complex are also incorporated during the assembly of the procapsid (Botstein *et al.*, 1973; Thomas & Prevelige, 1991). Holes present in the procapsid lattice allow the scaffolding protein to exit during the packaging of DNA through the portal vertex (King *et al.*, 1973; King & Casjens, 1974; Bazinet & King, 1985). The packaging of DNA is accompanied by the simultaneous expansion of the capsid

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

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lattice into the mature capsid which is characterized by a 50 Å increase in diameter, a change in shape from spherical to icosahedral, and the closure of the holes in the lattice (Earnshaw & Casjens, 1980; King & Casjens, 1974; Prasad *et al.*, 1993).

Low-resolution morphological images of the procapsid and mature capsid have been attained by low-angle X-ray scattering and image reconstruction of cryoelectron micrographs (Earnshaw *et al.*, 1976; Earnshaw & Casjens, 1980; Prasad *et al.*, 1993; Thumin-Commike *et al.*, 1996). It appears that the coat protein subunits exist in four quasi-equivalent conformations in the $T = 7$ procapsid: one conformation in the pentamers and three in the hexamers (Thumin-Commike *et al.*, 1996). An additional four conformations likely occur in the mature capsid through domain movements of coat protein as a result of DNA packaging (Prasad *et al.*, 1993). Coat protein presumably adopts all of the required conformations for the procapsid during assembly through conformational switching of the monomer subunits (Caspar, 1980; Rossman & Erickson, 1985).

To probe the information content that directs the folding and assembly in the amino acid sequence of coat protein, we are characterizing the folding and assembly of coat protein with amino acid substitutions that cause conditional-lethal phenotypes *in vivo*. Eighteen temperature-sensitive for folding (tsf) mutants in P22 coat protein have been isolated and characterized *in vivo* (Gordon & King, 1993). At restrictive temperatures, defects in the folding pathway produced by the tsf amino acid substitutions interfere with the production of viable phage by causing the newly synthesized polypeptides to form inclusion bodies, thereby preventing the subunits from reaching an assembly-competent conformation. The 18 tsf mutants can be rescued at the nonpermissive temperature *in vivo* by the overproduction of GroEL and GroES which are actively involved in ensuring the correct folding of the tsf mutants (Gordon *et al.*, 1994; W. S. Nakonechny and C. M. Teschke, unpublished data). *In vitro*, five of the tsf mutant polypeptides characterized thus far refold into soluble species which are stable dimers and trimers that are assembly-incompetent (Teschke & King, 1995; Galisteo *et al.*, 1995).

In contrast to the coat protein tsf mutants, the cs mutants are assembly-competent *in vivo* at a nonpermissive temperature (16 °C) and readily polymerize into procapsids (Gordon, 1993; B. Greene and J. King, unpublished data). Despite the ability of the three cs mutants to assemble at the restrictive temperature, the procapsids formed are unable to package DNA and are inhibited in their morphogenic development from maturing into the final capsid structure. Purified procapsids of two of the mutants, T10I and R101C, were found to be devoid of scaffolding protein even though they had been produced *in vivo* at a permissive temperature. In contrast, N414S retained its scaffolding protein with a slightly higher affinity than wild-type procapsids (Gordon, 1993; B. Greene and J. King, unpublished results; Teschke & Fong, 1996). Therefore, unlike the tsf mutants which are defective in polypeptide folding, the three cs mutants appeared to be defective in carrying out the subunit interactions necessary for phage morphogenesis.

In vitro the cs amino acid substitutions of T10I and R101C appear to play a role in disrupting the folded conformation of coat protein since the monomeric coat protein subunits of T10I and R101C are unable to initiate the proper interactions needed to assemble into a procapsid (Teschke & Fong, 1996). Refolded T10I and R101C have a significantly lower affinity for scaffolding protein and are defective in the initiation of assembly, whereas refolded N414S subunits are able to interact with scaffolding protein and are assembly-competent. In this paper, we have found that the three cs amino acid substitutions affect both the folding and the final conformation of the refolded coat protein subunits. These results are not consistent with the notion that these cs amino acid substitutions exert their effect by decreasing the hydrophobicity of the protein.

MATERIAL AND METHODS

Chemicals. Ultrapure GuHCl and urea were purchased from Schwartz-Mann ICN. bisANS was purchased from Molecular Probes. Lyophilized proteases on beads were obtained from Sigma. All other chemicals were reagent grade and were purchased from common sources.

Buffer. The buffer used in all of the experiments, with the exception of the circular dichroism experiments, was 50 mM Tris base, 25 mM NaCl, 2 mM EDTA, and 0.2% βME, adjusted to pH 7.6 with HCl. The βME was added to the buffer just prior to its use.

Purification of cs Coat Proteins. The cs coat proteins used in the refolding experiments were obtained from empty procapsid shell stocks that were prepared as previously described in Prevelige *et al.* (1988), Teschke and King (1993), and Galisteo *et al.* (1995). Briefly, *Salmonella typhimurium* cells were infected with bacteriophage P22 carrying a cold-sensitive mutation in gene 5 which codes for coat protein and amber mutations to prevent capsid maturation and cell lysis. As a result, the infected cells accumulated procapsids. Four hours after infection, the cells were collected by centrifugation and were resuspended in a small volume buffer. The cells were lysed by a freeze/thaw cycle, treated with RNase and DNase, and spun at 80 000 rpm in a RP80AT rotor in a Sorvall RC M120EX microultracentrifuge to pellet the procapsids. After the procapsids were suspended in buffer on a Nutator apparatus (Clay Adams, Inc.) at 4 °C overnight, the procapsids were passed over a Sephacryl 1000 column to remove smaller contaminating proteins and membranes. The scaffolding protein was removed from the procapsids by repeated extractions with 0.5 M GuHCl and subsequent centrifugation to pellet the empty procapsid shells. T10I and R101C were extracted once, while N414S and wild-type (WT) procapsids were extracted three times. The protein concentration was determined by unfolding the shell stocks in 6 M GuHCl and using an extinction coefficient of 0.957 mL mg^{-1} cm^{-1} at 280 nm (Teschke *et al.*, 1993). The purified empty procapsid shells were suspended in buffer and stored indefinitely as 4 mg/mL stocks at 4 °C.

Refolding of Coat Protein by Dialysis. Empty procapsid shells were unfolded by incubation with 5.25 M GuHCl and 0.2% βME at a final protein concentration of 0.5 mg/mL. After the shells were allowed to unfold for 30 min at room temperature, a time sufficient for complete dissociation of the shells and unfolding of the coat protein subunits (data not shown), the protein concentration was determined by the absorbance at 280 nm. Appropriate dilutions using 5.25 M GuHCl were made to correct for any small concentration differences. The denatured samples were loaded into the

microdialyzer (Gibco-BRL Life Technologies) and dialyzed with buffer for a period of 4.5−5 h and at a rate of approximately 30 mL/h. The buffer was maintained at 4 or 20 °C. The residual GuHCl concentration was determined by refractive index and was below 40 mM each time.

Native Gel Electrophoresis. Coat protein samples were refolded by dialysis at either 4 or 20 °C as described above. Samples for the native gel were prepared by combining a portion of refolded coat protein with 3X native gel sample buffer. The samples were run on a native gel that consisted of a 4.3% stacking gel (pH 8.3) and a 7.5% separation gel (pH 9.5) as described in Andrews (1986). The gel was run at a 10 mA constant current and a temperature of 4 °C. The protein bands were visualized by Coomassie stain.

SDS−PAGE. Coat protein samples were refolded by dialysis at either 4 or 20 °C as described above. SDS gel samples were prepared by combining refolded coat protein with a 3X SDS sample buffer which were heated at a temperature of 90 °C for 5 min. The samples were run on an SDS gel that had a 4.3% stacking gel and a 10% separation gel. The gel was run at 200 mV at room temperature and subsequently stained with Coomassie blue.

Kinetics of Refolding and Fluorescence Spectra. Procapsid shells were first dissociated and unfolded by combining the stock shells with a solution of buffered 6 M GuHCl and 0.2% βME for a final protein concentration of 1 mg/mL and a GuHCl concentration of 4.5 M GuHCl. Unfolding was allowed to proceed for 30 min at room temperature, a time sufficient for complete dissociation of the shells and unfolding of the coat protein subunits (data not shown). Refolding was initiated by rapidly injecting the unfolded protein into buffer for a 200-fold dilution so that the final protein concentration was 5 μg/mL and the residual GuHCl concentration was 225 mM. The excitation wavelength (SLM Aminco-Bowman 2 fluorescence spectrophotometer) was 280 nm; the emission wavelength was 340 nm, and both the excitation and emission band-pass were set at 4 nm. The cuvette was maintained at 7 or 20 °C with a water bath. Changes in fluorescence were measured over 3600 s (20 °C) or 10 800 s (7 °C). The temperature 7 °C was chosen because the water bath was able to maintain that temperature in the cuvette holder relatively easily. Alternatively, the refolding was done on ice for 4−5 h. At the end of each refolding experiment, an emission scan from 310 to 400 nm was performed. A kinetic analysis of the data was done using Kaleidagraph (Abelbeck) or Igor Pro (WaveMetrics). Relaxation times were calculated by using the formula for a first-order decay with two exponentials which yielded the best fit to the data.

Kinetics of Refolding in the Presence of bisANS and Fluorescence Spectra. A stock solution of bisANS was prepared in buffer, and the concentration of this solution was determined by using the extinction coefficient of 16 790 L mol^{-1} cm^{-1} at 385 nm (Farris *et al.*, 1978). Empty procapsid shells were denatured and unfolded as above. Refolding was initiated in the SLM Aminco-Bowman 2 fluorescence spectrophotometer by rapidly injecting the unfolded coat protein into buffer containing 2 μM bisANS to give a 200-fold dilution such that the final protein concentration was 5 μg/mL. The fluorescence spectrophotometer was set as follows: excitation wavelength, 390 nm; emission wavelength, 495 nm; and band-passes, 4 nm. A constant temperature of 7 or 20 °C was maintained by a water bath-controlled cuvette holder. Alternatively, the refolding was done on ice for 4−5 h. At the end of each folding reaction, an emission scan from 425 to 625 nm was performed. Relaxation times of folding monitored by bisANS fluorescence were calculated with Kaleidagraph (Abelbeck) or Igor Pro (WaveMetrics) using the formula for a first-order decay with two exponentials.

Circular Dichroism of Refolded Coat Protein. Samples were prepared in the microdialyzer at 4 or 20 °C as described above except that 20 mM phosphate at pH 7.6 was used in the dialysis. The protein concentrations were determined using the extinction coefficient for coat protein of 0.957 mL mg^{-1} cm^{-1} at 280 nm (Teschke & King, 1995). Aggregates in the refolded samples were removed by centrifugation for 5 min in a microfuge at 4 °C prior to adjusting the protein concentration. The spectra, the average of nine scans, were taken with a Jasco J-710 circular dichroism spectrophotometer thermostated at 4 or 20 °C from 250 to 190 nm with the following settings: a scan rate of 20 nm/min, a response time of 4 s, a band width of 2 nm, and a step resolution of 0.1 nm. A 1 mm path length cell was used.

Proteolysis of Refolded Coat Protein. Lyophilized chymotrypsin and V8 protease attached to agarose beads were rehydrated and washed three times with cold buffer, and a final 1:1 slurry of beads/buffer was kept on ice prior to use. Refolded coat protein was prepared by dialysis at 4 or 20 °C as described except that concentration differences were corrected after dialysis. Aggregates in the refolded samples were removed by centrifugation for 5 min in a microfuge at 4 °C prior to adjusting the protein concentration. For each digest, a 50 μL sample of refolded coat protein was combined with 5 μL of the 1:1 slurry in a microcentrifuge tube and placed on a Nutator apparatus at 4 °C or at room temperature (∼20 °C). At the end of the digestion period, the tubes were pulsed for 5 s in a microfuge to pellet the beads. The supernatants were combined with 3X SDS sample buffer, run on 15% SDS gels, and silver stained (Rabilloud *et al.*, 1988). Digestion periods are described in the figures. The band of full length coat protein was quantified using a Molecular Dynamics computing densitometer.

GroEL/GroES Plating Experiment. S. typhimurium cells transformed with pOF39 which carries the GroEL/S operon behind its own promoter or the control plasmid, pBR322, were infected with each of the cs mutants and WT phage at 16, 20, and 30 °C (Fayet *et al.*, 1986). After the infected cells were allowed to grow overnight, the relative plating efficiency was determined by comparing the titer on each of the plasmid-bearing strains at 16 and 20 °C to the titer at 30 °C. The amount of GroEL produced at 16 °C in cells carrying pOF39 was compared to that of cells carrying pBR322 by SDS−PAGE.

RESULTS

We have previously found that the procapsid lattice of all three cs mutants formed *in vivo* at a permissive temperature showed perturbations in stability and ability to expand, showing that the cs amino acid substitutions affect morphogenesis even at permissive temperatures (Teschke & Fong, 1996). When we investigated the assembly competence of the cs coat polypeptides, we found that they formed monomers when refolded *in vitro* at either 4 or 20 °C, but only the monomers of N414S were assembly-competent.

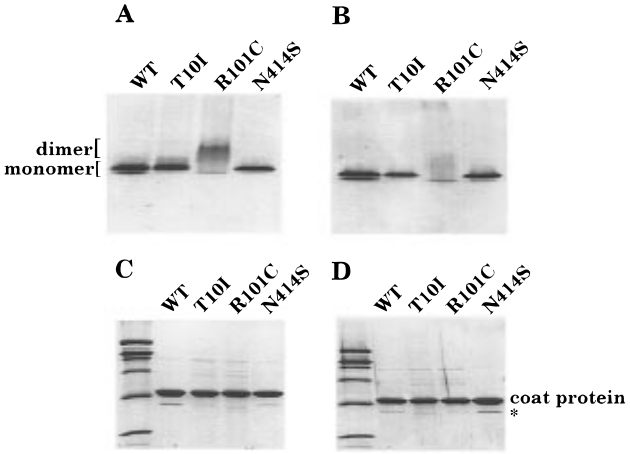

FIGURE 1: Native and SDS−polyacrylamide gel electrophoresis of refolded WT and cs mutant coat proteins. The coat proteins were refolded from 5.25 M GuHCl by dialysis at either 4 °C (A and C) or 20 °C (B and D). Native gels are shown in panels A and B and SDS gels in panels C and D. The molecular mass markers are shown in the left lane of panels C and D and from the top have M_rs of 200, 116, 97.4, 66, 45, 33, and 21.5 kDa. The * marks the position of the scaffolding protein, a minor contaminant in the coat protein preparations.

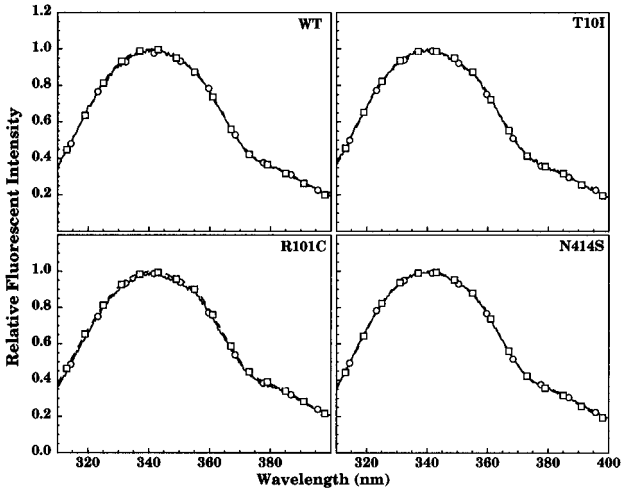

FIGURE 2: Tryptophan fluorescence emission spectra of refolded WT and cs mutant coat proteins. The emission scan was performed 1 h after the initiation of folding at 20 °C (○) or after 4−5 h on ice (□) from 4.5 M GuHCl by rapid dilution. Fluorescence excitation was set at 280 nm, and the emission was monitored from 310 to 400 nm. The final protein concentration was 5 μg/mL. The data were normalized so that the point of highest intensity is 1.0. The panels are labeled with the protein species.

Here we have examined the effects of three cs amino acid substitutions on the folding pathway and the folded conformation of coat protein monomers to determine the reason the cs amino acid substitutions caused different effects when refolded *in vitro*.

Refolding of the cs Coat Proteins. To examine the effect of temperature on the efficiency of folding, unfolded coat polypeptides were refolded by dialysis at either 4 or 20 °C and run on a native gel to determine if there were any changes in the yield of the monomer (Figure 1A,B). An SDS gel was run to ensure that the loaded protein concentrations were the same (Figure 1C,D). At both 4 and 20 °C, T10I and N414S folded into a monomeric species that migrated to the same position as the upper band of the two species of WT coat protein monomers (Figure 1A,B). In addition, the intensities of the monomer bands of T10I and N414S were comparable to that of WT coat protein at each temperature, suggesting that they folded with similar efficiencies. The folding of R101C, however, was inefficient relative to those of the other mutants since the intensity of the monomer band at each temperature was significantly diminished. The monomeric species of R101C migrated further into the native gel than the other mutants probably due to a change in the charge to mass ratio brought about by the cs amino acid substitution from arginine to cysteine. At 4 °C, R101C exhibited an increased propensity to form dimers in addition to other higher oligomeric states (Figure 1A). On the other hand, at 20 °C (Figure 1B), R101C showed very little of the dimeric species and only a faint uniform smear of various oligomeric states present above the monomer band. Although there were noticeable differences in the concentration of oligomers at the two temperatures, the yield of monomeric R101C was comparable. The amount of monomeric R101C that remained in the refolding reaction was likely at a concentration below the critical aggregation concentration and was therefore able to refold rather than aggregate. Other experiments have shown that there is a great deal of variability in yield, especially at 4 °C (data not shown).

Fluorescence Spectra of Refolded cs Coat Proteins. One approach for probing for conformational changes in refolded coat protein is to examine the environmentally-sensitive intrinsic fluorescence of its six tryptophans (Eppler *et al.*, 1991; Teschke & King, 1993, 1995). Shifts in the wavelength of the emission maximum are an indication of a conformational change in the folded monomers. In five of the tsf mutants of coat protein thus far studied, the fluorescence emission spectra did not show dramatic changes in the environment of the tryptophans, indicating that the tsf amino acid substitutions only altered the hydrophobic core slightly even though the folded conformation of each tsf mutant was drastically affected (Teschke & King, 1995).

To examine whether the tryptophans were in the same environment for each of the three cs mutants, a fluorescence emission scan was done on protein folded by rapid dilution at 20 °C or on ice (Figure 2). Regardless of the temperature of refolding, the fluorescence emission spectrum of each of the mutants was identical, indicating that the cs amino acid substitutions do not affect the final environment of the six tryptophan residues in the folded structure of coat protein.

In addition to conformational information that can be obtained from a scan of the fluorescence emission of tryptophan residues, the extrinsic fluorescence of a marker molecule such as bisANS can be used to probe for conformational differences in the folded structure of a protein. In the folded structure of WT coat protein monomers, a single bisANS molecule binds to a hydrophobic region on the surface of the protein which is important for assembly (Teschke & King, 1993; Teschke *et al.*, 1993). Since the fluorescence of a molecule is dependent upon the environment, differences in the fluorescence of bisANS would indicate a change in bisANS binding to coat protein. In the tsf mutants, for example, the fluorescence emission spectra were similar except for a slight red shift relative to that of bisANS bound to WT coat protein, indicating that the environment of bisANS in the final structure of the tsf mutants must be slightly different than that in WT coat protein (C. M. Teschke, unpublished data). When the cs

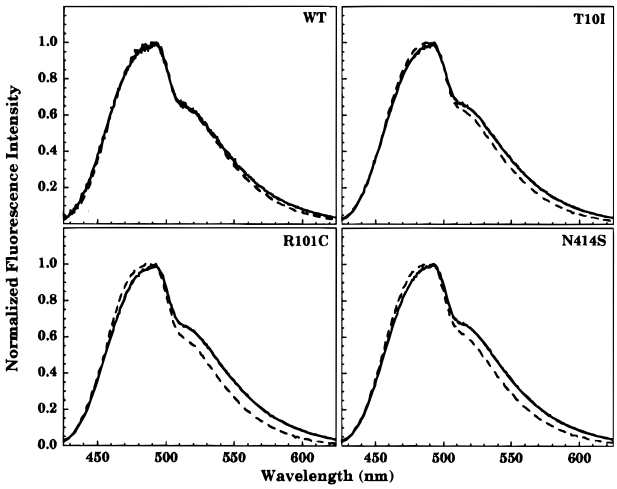

FIGURE 3: Fluorescence emission spectra of refolded WT and cs mutant coat protein in bisANS. The emission scan was performed 1 h after the initiation of folding at 20 °C (—) or after 4−5 h on ice (- - -) from 4.5 M GuHCl by rapid dilution. The buffer contained 2 μM bisANS. Fluorescence excitation was set at 390 nm, and the emission was monitored from 425 to 625 nm. The final protein concentration was 5 μg/mL. The data were normalized so that the point of highest intensity is 1.0. The panels are labeled with the protein species.

mutants were folded at 20 °C in the presence of 2 μM bisANS, identical spectra were obtained (Figure 3, solid line). However, when refolded at 4 °C (Figure 3, dashed line), each of the cs mutants showed a blue shift compared to the spectra taken at 20 °C, indicating that the bisANS binding site must be more protected from solvent. WT coat protein showed no significant spectral shifts regardless of the temperature of folding. Therefore, when each mutant was folded at 4 °C, the conformation of the folded state must have been altered so that the bisANS binding pocket was perturbed.

Kinetics of Refolding of the cs Coat Proteins at 20 and 7 °C. What is observed in a refolding experiment of coat protein when the denaturant is diluted by manual mixing is the folding from a long-lived folding intermediate, rather than from the unfolded protein, to the final folded state (Teschke & King, 1993, 1995). Effects of amino acid substitutions on the formation of the long-lived intermediate cannot be determined in these experiments because the formation of the intermediate occurs in the dead time of the manual mixing experiment. Within these limitations, we have investigated the folding of the cs mutants to determine if there was any effect on the folding from the long-lived intermediate.

To determine the effects of the cs amino acid substitutions on the kinetics of folding, the changes in the intrinsic fluorescence of the six coat protein tryptophans were monitored as a function of time in triplicate at 20 and 7 °C. The three cs mutants fold at 20 °C with the same two-phase kinetics as WT coat protein (Table 1). As typically observed in the folding of coat protein, the fluorescence decreased with time in a curve best fit to a first-order decay with two exponents which account for all the fluorescence change (Teschke & King, 1993, 1995). In each case, both the fast and slow relaxation times in the folding of the cs mutants were essentially identical to that of WT coat protein except for the fast phase in the folding of R101C which was slightly slower than WT folding. One notable difference between the folding of WT and the cs mutant proteins was that the slow relaxation accounted for a larger portion of the total

Table 1: Relaxation Times of Folding of the cs Mutant Coat Proteins from GuHCl at 20 °C[a]

coat protein	τ_1 (s)	τ_2 (s)	fraction τ_1	fraction τ_2
WT	189 ± 96	2132 ± 1033	0.58 ± 0.15	0.42 ± 0.15
T10I	198 ± 49	1903 ± 1336	0.39 ± 0.06	0.61 ± 0.06
R101C	516 ± 224	2355 ± 1678	0.46 ± 0.11	0.54 ± 0.11
N414S	273 ± 69	2508 ± 402	0.43 ± 0.10	0.57 ± 0.10

[a] The relaxation times of folding were determined by fitting the data to a first-order decay with two exponentials.

Table 2: Relaxation Times of Folding of the cs Mutant Coat Proteins from GuHCl and 2 μM bisANS at 20 °C[a]

coat protein	τ_1 (s)	τ_2 (s)	fraction τ_1	fraction τ_2
WT	99 ± 53	591 ± 51	0.29 ± 0.03	0.71 ± 0.03
T10I	25 ± 6	488 ± 135	0.30 ± 0.09	0.70 ± 0.09
R101C	47 ± 22	662 ± 34	0.24 ± 0.06	0.76 ± 0.06
N414S	25 ± 12	440 ± 38	0.20 ± 0.06	0.80 ± 0.06

[a] The relaxation times of folding were determined by fitting the data to a first-order decay with two exponentials.

change in fluorescence in the cs mutants, indicating a change in the folding mechanism. When the folding of WT and the cs mutants was investigated at 7 °C, we found the rate of refolding was so slow (greater than 9000 s) that relaxation times could not be accurately measured. This large change in relaxation time with the decrease in temperature suggests that the folding of coat protein may be intrinsically sensitive to decreases in temperature, an indication of the difficulty of burying hydrophobic residues in the folding of coat protein.

In addition to the intrinsic fluorescence of tryptophan residues, the extrinsic fluorescence of an added dye can be used to examine the kinetics of folding *in vitro*. bisANS binds to the long-lived intermediate detected in the folding of coat protein (10 bisANS molecules/intermediate). As folding proceeds, the number of bound bisANS reaches one molecule per folded monomer (Teschke & King, 1993). In the analysis of folding where bisANS was used as a probe, it was observed that five of the tsf mutants folded with similar kinetics (Teschke & King, 1995). To determine the effect of the cs amino acid substitutions on the folding of the long-lived folding intermediate, the three cs mutants were refolded from GuHCl by rapid dilution into buffer containing 2 μM bisANS at 20 or 7 °C. The change in bisANS fluorescence was monitored over time, and relaxation times were calculated from triplicate experiments (Table 2). As seen in the kinetics of folding monitored by tryptophan fluorescence, the kinetics of folding monitored by bisANS exhibited two phases. However, the fast and slow relaxation times for T10I and N414S were slightly faster than that of WT coat protein. Consistent with the analysis of folding by tryptophan fluorescence, the slow relaxation of R101C was slower than that of WT coat protein. The fast relaxation of R101C was slightly slower than that of T10I and N414S. The fraction of the fluorescence change accounted for by each relaxation did not change. Once again, when the folding was investigated at 7 °C, the kinetics were too slow to be measured accurately. These data suggest that the changes seen in the kinetics of folding monitored by the intrinsic fluorescence of tryptophans were global, involving hydrophobic packing, rather than specific for the positioning of the tryptophans within the core of coat protein, and that the cs amino acid substitutions alter the folding pathway of coat protein.

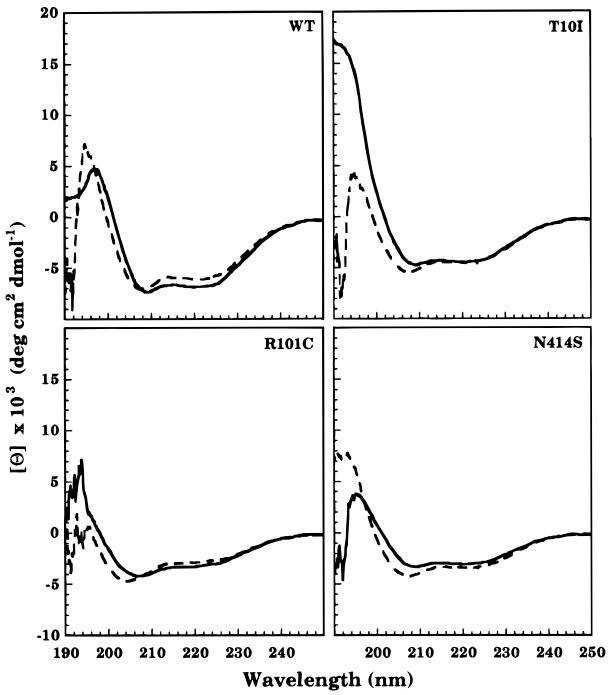

FIGURE 4: Secondary structure of refolded WT and cs mutant coat proteins monitored by circular dichroism. The proteins were refolded by dialysis at either 20 °C (—) or 4 °C (- - -). The experiment was done as described in Materials and Methods. The panels are labeled with the protein species.

Affect of the cs Amino Acid Substitutions on Secondary Structure of the Refolded Monomers. The data described above suggest that there may be changes in the folding of the cs coat proteins. To elucidate the effects of the cs amino acid substitutions on the folded conformation, the secondary structure of the cs coat protein monomers was examined after refolding by dialysis at 4 and 20 °C (Figure 4). To obtain reasonably good spectra, the buffer was changed from our standard Tris/NaCl/EDTA/βME buffer at pH 7.6 to 20 mM phosphate at pH 7.6. To be certain that the spectra taken in this buffer reflected the structure we obtained when using our standard buffer, we refolded at 4 °C in the phosphate buffer and did assembly reactions by the addition of scaffolding protein to polymerize procapsids. Both WT coat protein and N414S were as assembly-competent as when folded in our standard buffer (data not shown) (Teschke & Fong, 1996). Additionally, the limited proteolysis experiments described below were done on WT and cs coat protein samples refolded in phosphate buffer, and the results were the same at those done in our standard buffer (data not shown). Refolding in phosphate buffer, however, resulted in an increase in trimers in the folded WT coat protein as well as in the mutant protein samples (data not shown). Since WT coat protein and N414S were as assembly-competent as when they were refolded in our standard buffer, we were confident that the spectra would reflect any changes in the conformation of the cs coat proteins. We also took spectra in our standard buffer, which absorbs too strongly to obtain good quality spectra below 205 nm, to be sure that changes seen in the spectra of the cs mutants in phosphate buffer were also seen when the proteins were refolded in our standard buffer (data not shown). The spectra obtained in phosphate buffer were good to 195 nm. Below 195 nm, the spectra became noisy most likely due to the residual GuHCl which was 15−20 mM. As observed previously, the spectrum of WT coat protein folded at 20 °C showed peaks at 209 and 221 nm with negative ellipticities of −7300 and −6800 deg cm^2 dmol^{-1}, respectively (Figure 4, solid line). As before, these data were not fit well by the standards of Chang *et al.* (1978; Teschke *et al.*, 1993). The spectra of the cs proteins folded at 20 °C all showed decreases in negative ellipticity at both 209 and 221 nm. The spectrum of N414S showed the largest decrease in negative ellipticity at 209 nm to −3300 deg cm^2 dmol^{-1}, while the spectra of T10I and R101C had negative ellipticities of −4700 and −4200 deg cm^2 dmol^{-1}, respectively, at 206 nm. At 221 nm, the spectra of N414S and T10I exhibited decreases in negative ellipticities to −3000 and −4400 deg cm^2 dmol^{-1}, respectively. The spectrum of R101C had a negative ellipticity at 218 nm of −3300 deg cm^2 dmol^{-1}. The spectrum of R101C also showed a change in shape which may indicate a change in the amount of random coil compared to those of the other mutants, whereas T10I showed an increased signal at lower wavelengths that might be indicative of an increase in α-helix. These changes are consistent with a decrease in the amount of secondary structure present in refolded subunits. When the proteins were refolded at 4 °C (Figure 4, dashed line), small shifts were seen in the spectra, especially at the 208 nm peak. Some changes were also observed in the WT spectrum, but the differences were somewhat more substantial among the mutants. We conclude from these data that the cs amino acid substitutions affect the folding of coat protein into the correct conformation.

Changes in the Surface Structure of the cs Coat Protein Monomers. To probe for tertiary structure changes in the three cs mutants, limited proteolysis was performed using either chymotrypsin or V8 protease. If the folded coat protein monomers of the three cs mutants exist in the same tertiary conformation, the digestion would yield the same cleavage patterns and overall sensitivity to proteolysis. The coat protein preparations are at least 90−95% pure (Figure 1C,D), and several of the peptides from the chymotrypic digest were N-terminally sequenced and were found to be coat protein fragaments (data not shown); therefore, any differences in proteolysis patterns were due to changes in the accessibility of the protease to cleavage sites. WT and the cs mutants were refolded at 4 or 20 °C by dialysis in our standard buffer to compare changes in tertiary structure as a result of folding at each temperature. In these experiments, we chose times so that the extent of proteolysis did not exceed ∼50% of the total protein added to the reaction so that we observed the earliest cleavages and would be more likely to detect changes in conformation of the full length protein rather than the proteolytic sensitivity of the clipped fragments. Additionally, we chose both a nonspecific protease, chymotrypsin, and V8 protease, a specific protease, to determine if overall conformational changes had occurred or if the changes were more localized.

In the protease digests of the three cs mutants, changes in sensitivity to protease were revealed (Figures 5−8). In the proteolysis of WT and the cs mutants folded at 20 °C with chymotrypsin (Figures 5B and 6B), little changes in peptide patterns or in the rate of digestion by the protease were seen. However, when the proteins were refolded at 4 °C (Figures 5A and 6A), differences in the accessibility to chymotrypsin were seen. N414S was more resistant to chymotrypsin compared to WT coat protein, while T10I and R101C were

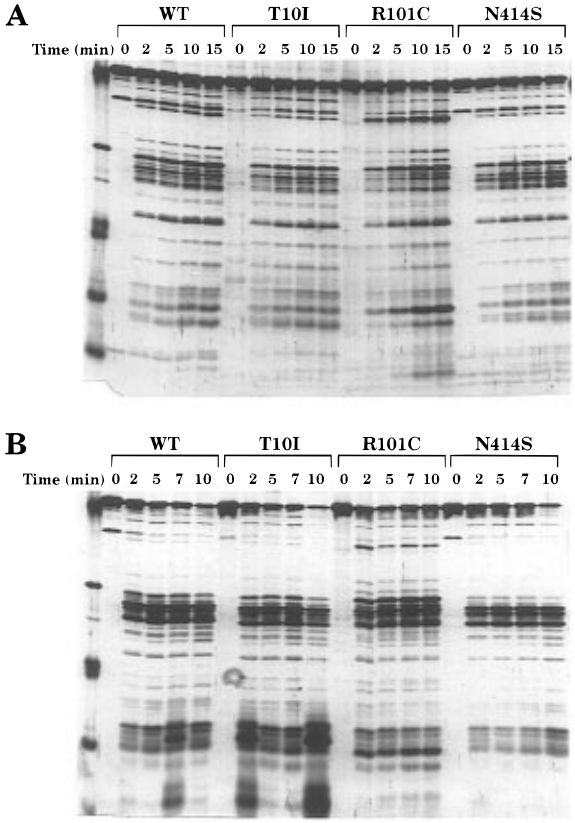

FIGURE 5: Proteolysis of WT and cs mutant coat proteins folded at 4 °C (A) or 20 °C (B) using chymotrypsin protease attached to agarose beads. The coat protein peptides were run on 15% SDS−polyacrylamide gels. The molecular mass markers are shown in the left lane and from the top have M_rs of 45, 33, 21.5, 14.4, and 6.5 kDa.

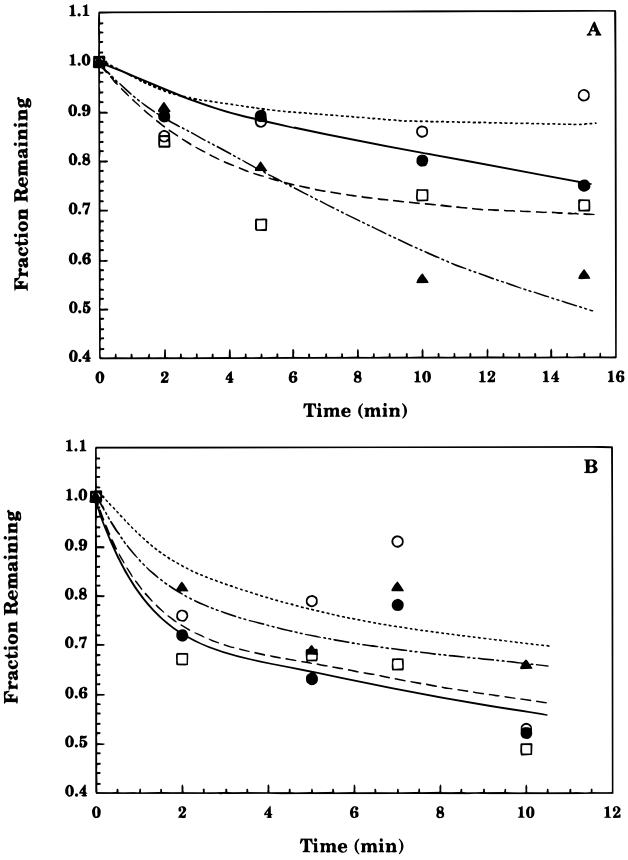

FIGURE 6: Time course of digestion of full length coat protein by chymotrypsin. The amount of full length coat protein was quantified and the fraction remaining at each time determined. The lines are drawn to aid the eye. Panel A shows the time course for proteins folded at 4 °C and panel B for the proteins folded at 20 °C. The symbols are as follows: WT coat protein (●, —), T10I (□, - - -), R101C (▲, -···-), and N414S (○, ···).

more sensitive to the protease. The peptide fragments seen on the SDS gels were similar, indicating that there were no new cleavage sites. When WT and cs coat proteins refolded at 20 °C were treated with V8 protease (Figures 7B and 8B), again, changes in sensitivity to the protease were seen. N414S was more resistant to the V8 protease than WT coat protein, while T10I and R101C showed little difference in the rate of proteolysis. When the proteins were refolded at 4 °C (Figures 7A and 8A), both N414S and T10I exhibited resistance to V8 protease while R101C showed sensitivity to V8 protease comparable to that of WT coat protein. N414S was much less sensitive to digestion by V8 protease, suggesting that V8 cleavage sites might be hidden in the folded conformation of this mutant. Again, no major differences in peptide fragments were seen on the SDS gels. The fact that there were no differences in the peptide fragment patterns on the SDS gels suggests that the major elements of secondary structure may be folded correctly but that the packing of those elements has been affected by the cs amino acid substitutions.

GroEL/GroES Plating Experiments. Folding coat polypeptides have the potential to interact with molecular chaperones such as GroEL and GroES. GroEL and GroES have been shown to interact with the coat tsf polypeptides during folding *in vivo* (Gordon *et al.*, 1994; W. S. Nakonechny and C. M. Teschke, unpublished results). Tsf mutants growing on cells that overproduce GroEL and GroES are rescued at nonpermissive temperatures and produce viable phage, while in the absence of GroEL and GroES overproduction, tsf mutants form inclusion bodies *in vivo*. Ostensibly, GroEL and GroES recognize a folding defect, interact with the defective folding intermediate, and rescue the tsf mutants from the incorrect folding pathway.

To examine whether the cs amino acid substitutions produce changes in the folding pathway that are recognized by GroES and GroEL, the cs mutants were grown on *S. typhimurium* cells that overproduce GroEL and GroES (Figure 9B). Below the permissive temperature of 20 °C, there was a precipitous drop in plating efficiency for each of the three cs mutants even in the presence of overproduced GroEL and GroES (Figure 9A). In contrast, WT phage did not suffer a loss in plating efficiency even at 16 °C. The overproduction of GroEL and GroES therefore does not rescue the folding of the cs mutants.

DISCUSSION

Previously, we had found that the folded coat protein monomers of T10I and R101C were assembly-incompetent and were defective in their ability to correctly interact with scaffolding protein to initiate the assembly process (Teschke & Fong, 1996). One possibility was that both T10I and R101C folded into a different conformation than WT coat protein or N414S. Another possibility was that the threonine at position 10 and the arginine at position 101 were directly involved in the binding of scaffolding protein. We also found that the capsid lattice formed from N414S coat protein exhibited an increased affinity for scaffolding protein relative

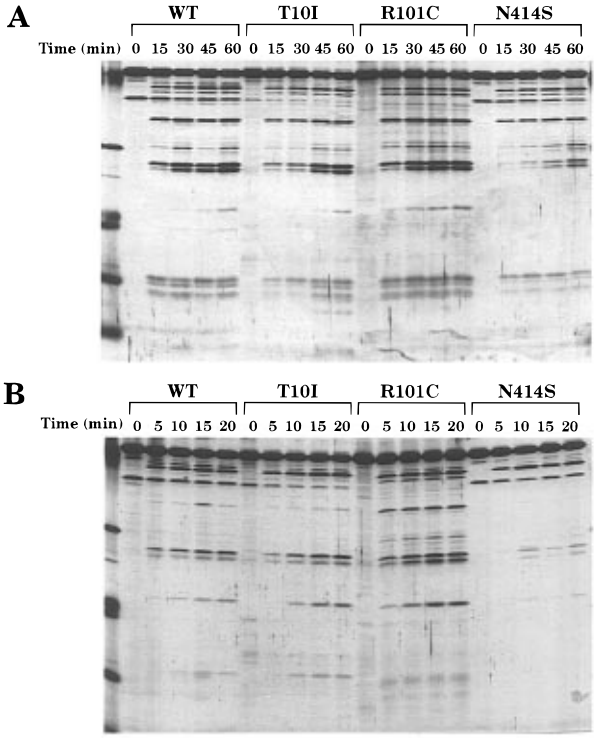

FIGURE 7: Proteolysis of WT and cs mutant coat protein folded at 4 °C (A) or 20 °C (B) using V8 protease attached to agarose beads. The coat protein peptides were run on 15% SDS−polyacrylamide gels. The molecular mass markers are shown in the left lane and from the top have M_rs of 45, 33, 21.5, 14.4, and 6.5 kDa.

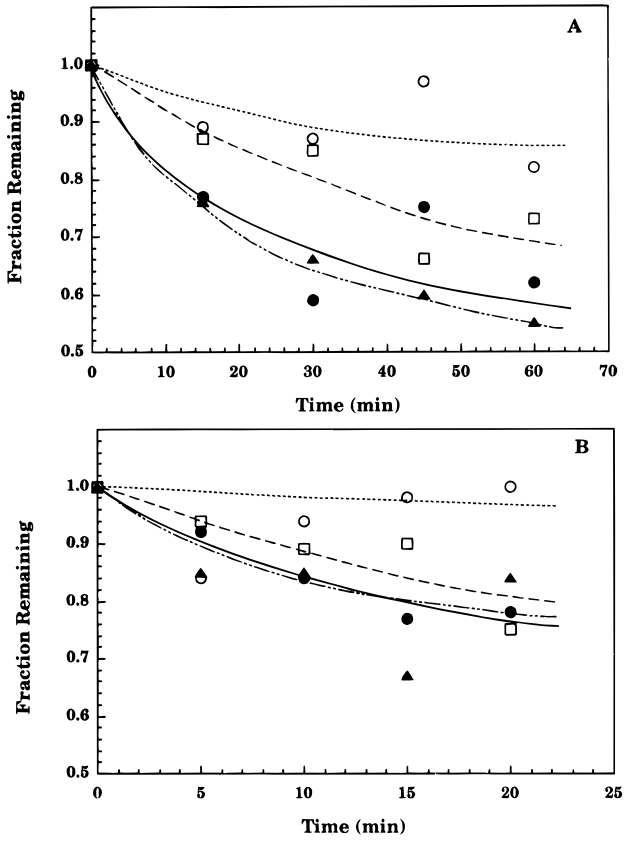

FIGURE 8: Time course of digestion of full length coat protein by V8 protease. The amount of full length coat protein was quantified and the fraction remaining at each time determined. The lines are drawn to aid the eye. Panel A shows the time course for proteins folded at 4 °C and panel B for the proteins folded at 20 °C. The symbols are as follows: WT coat protein (●, —), T10I (□, - - -), R101C (▲, -···-), and N414S (○, ···).

to WT capsids, an indication of a change in conformation of the subunits. The lattices of procapsids formed from T10I and R101C were also affected by their amino acid substitutions (Teschke & Fong, 1996). Here we have examined the folding and conformation of the refolded coat protein subunits to determine how the cs amino acid substitutions play a role in the folding process of coat protein *in vitro*.

Unlike the tsf mutants that fold into dimers and trimers *in vitro*, each of the three cs mutants folded from GuHCl by dialysis into soluble monomeric species. The folding of R101C into monomers was found to be substantially lower in efficiency than the other two mutants at both 4 and 20 °C. To probe for differences in the folding pathway of the three cs mutants, the kinetics of folding were monitored by the change in the intrinsic fluorescence of the tryptophans. In each of the three cs mutants, relaxation times were within experimental error except for the fast relaxation of R101C which appeared to be slightly slower than that observed in the folding of WT coat protein. The portion of the total fluorescence amplitude that occurred in the slow relaxation increased in the folding of the mutants, suggesting that the cs amino acid substitutions changed the folding mechanism so that the slow pathway became dominant. Perhaps folding along the slow pathway allows for more errors in packing of secondary structure elements to occur. When the kinetics of folding were examined by the changes in the extrinsic fluorescence of bisANS, the fast and the slow relaxation times in the folding of T10I and N414S were faster than those in the folding of WT coat protein, whereas R101C had slower rates of folding in bisANS. Perhaps these changes in the rate of sequestration of hydrophobic regions are indicative of the errors made in the folding of the cs coat proteins.

The fluorescence emission spectra indicated that the final environment of the tryptophan residues in the folded structure of the cs mutant monomers was identical to that of WT coat protein, regardless of the temperature of folding. Since tryptophan residues are likely to be sequestered into the hydrophobic interior of coat protein, the identical fluorescence emission spectra of the three cs mutants and WT coat protein suggest that the cs amino acid substitutions do not affect the formation of the core, just the rate at which it forms, even though the secondary structure of each of the mutants has been affected by the cs amino acid substitutions as observed by changes in the CD spectra. Presumably, the cs amino acid substitutions affect the folding of surface regions of coat protein rather than the core of coat protein. In agreement with this notion, when the cs mutants were folded at 4 °C, changes in the bisANS fluorescence spectra were seen that suggested that there were some alterations in the surface hydrophobic patch.

To probe for changes in tertiary structure and surface morphology of the cs mutants, limited proteolysis of coat protein was performed with chymotrypsin and V8 protease. The observed differences in sensitivity to protease were consistent with changes in surface structure in all of the cs mutants. The folded monomers of N414S are assembly-competent (Teschke & Fong, 1996), yet differences in its sensitivity to proteolysis and CD spectrum suggest that there are alterations in the conformation of this mutant as well. These data also suggest that different regions or loops must

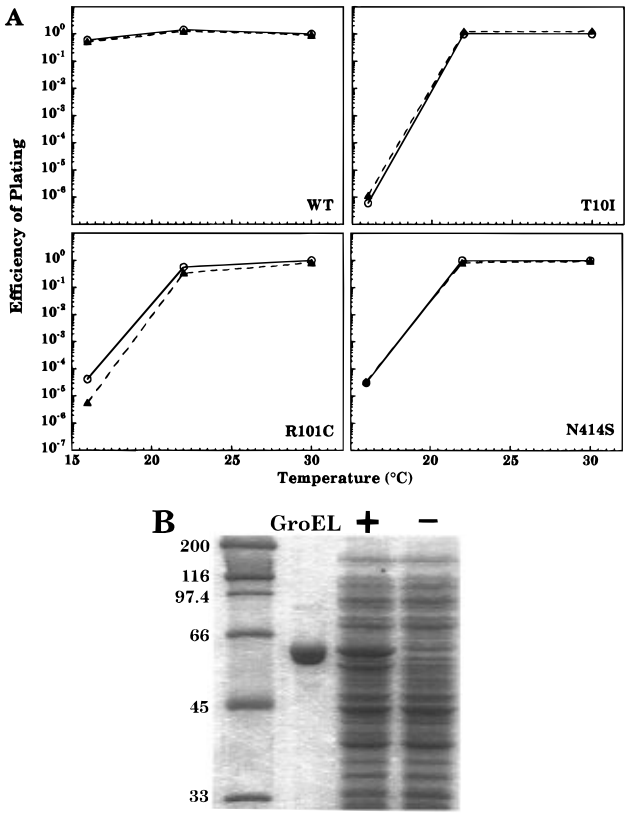

FIGURE 9: Efficiency of plating in the presence and absence of GroEL and GroES overproduction. (A) *S. typhimurium* carrying the plasmid pOF39 (○, solid line), which expresses the genes encoding GroEL and GroES, and the control plasmid pBR322 (▲, dashed line) were infected with WT and the cs coat protein mutant phage at 16, 20, and 30 °C. Plating efficiency was calculated as described in Materials and Methods. The overproduction of GroEL and GroES was confirmed at 16 °C by SDS−polyacrylamide gel electrophoresis (B). The proteins produced by pOF39 (+) and pBR322 (−) bearing cells were visualized on a 10% SDS−polyacrylamide gel. The molecular masses of the SDS gel markers in kilodaltons are given on the left side of the panel.

be affected by the substitution of N414S as compared to T10I or R101C. Considering that the conformation of N414S is substantially affected by its amino acid substitution suggests that the amino acids at positions 10 and 101, or the amino-terminal region, are directly involved in the interaction of coat protein with scaffolding protein. Each of the procapsid lattices was affected by the amino acid substitutions (Teschke & Fong, 1996) which is consistent with the fact that the conformation of the folded subunit of each cs mutant was altered by the amino acid substitution.

Why do T10I and R101C fold into assembly-incompetent monomers *in vitro* but assemble into procapsids *in vivo*? One possible answer is that a molecular chaperone rescues the two cs mutants from defective folding *in vivo*. The tsf mutants have been shown to be rescued by overproduction of the molecular chaperones GroEL and GroES at nonpermissive temperatures. Presumably, GroEL and GroES recognize the folding defect, interact with the defective folding intermediate, and rescue the mutant polypeptides from the incorrect folding pathway. When the cs mutants were grown in cells overproducing GroEL and GroES, the three mutants were not rescued at the nonpermissive temperatures, suggesting that the folding defects associated with the cs amino acid substitutions must not be recognized by GroEL and GroES. The isolation and characterization of the chaperone(s) involved in the *in vivo* assembly competence of T10I and R101C may provide additional insight into the defects in their folding pathway. Another explanation for the capability of T10I and R101C to assemble *in vivo*, but not *in vitro*, is that a phage protein such as one of the three pilot proteins or the portal protein complex may be needed. These proteins are ordinarily not essential for procapsid assembly *in vivo* (Bazinet & King, 1988; Thomas & Prevelige, 1991), but the cs substitutions may make them vital for assembly. A search for extragenic second site suppressors could help resolve the possibilities since such suppressors would be expected to be isolated if there is an important interaction occurring during assembly *in vivo*.

The cs amino acid substitutions have revealed new roles for particular amino acid residues in folding of coat protein monomers. The results discussed here suggest that the amino acid residues identified by the cs mutants are important for the conformation of the monomeric state, the folding to that state, and the protein−protein interactions used during assembly of capsids. These mutants are different from the tsf coat protein mutants where the amino acid substitution causes incorrectly associated folding intermediates leading to an alternate stable state comprised of dimers and trimers. The folding of a protein that is destined to reside in a large multisubunit complex, such as the P22 procapsid, must be delicately balanced so that assembly will not occur prematurely and only with the assistance of scaffolding protein. We continue to identify conditional-lethal mutants to dissect the amino acid residues that are critical for both the folding and assembly of this complex protein.

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