Folding Defects Caused by Single Amino Acid Substitutions in a Subunit Are Not Alleviated by Assembly†

Carole M. Capen and Carolyn M. Teschke*

Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut 06269-3125

Received August 20, 1999; Revised Manuscript Received October 26, 1999

ABSTRACT: Significant stabilization of a protein often occurs when it is assembled into an oligomer. Bacteriophage P22 contains 420 monomers of coat protein that are stabilized by the assembly and maturation processes. The effects of eight single amino acid substitutions in coat protein that each cause a temperature-sensitive-folding defect were investigated to determine if the conformational differences previously observed in the monomers could be alleviated by assembly or maturation. Several techniques including differential scanning calorimetry, heat-induced expansion, urea denaturation, and sensitivity to protease digestion were used to explore the effects of the amino acid substitutions on the conformation of coat protein, once assembled. Each of the amino acid substitutions caused a change in the conformation as compared to wild-type coat protein, observed by at least one of the probes used. Thus, neither assembly nor expansion entirely corrected the conformational defects in the monomeric subunits of the folding mutants.

Most proteins contain sufficient information in their linear amino acid sequence to ensure the folding into the correct three-dimensional structure under the appropriate conditions (*1*). For some time many have believed that thermodynamics, i.e., the axiom that a protein will fold to its lowest free energy, outweighed any kinetic considerations. In recent studies, however, the idea of several energy minima being available to polypeptides during folding has been emphasized, with the deciding factor between alternate conformations being determined by a kinetic partitioning between the available conformations (*2*−*5*). Some proteins that are demonstrably aggregation-prone require molecular chaperones to assist in their proper folding as they are by themselves unable to avoid pathways leading to nonnative associations (*6*). The further elucidation of the mechanisms involved in controlling the fate of newly synthesized proteins is of great importance to the biotechnology industry, where proteins expressed in heterologous hosts are often prone to aggregation and thus difficult to purify in their native, active conformation (*7*−*9*). Additionally, many human diseases are caused by the misfolding and aggregation of proteins (*10*−*18*). For multimeric proteins, folding is often linked with oligomerization, as subunit interactions provide additional stability to each monomer (*19*−*24*). Here we examine P22 bacteriophage coat protein, a monomer that interacts with other subunits to achieve increased structural integrity (*25, 26*). During the assembly and expansion processes, coat protein must be able to adopt one of seven quasi-equivalent conformations leading to at least four unique conformations (*27, 28*).

P22, a bacteriophage of *Salmonella typhimurium*, has been used extensively as a source of model proteins to investigate the folding process (*29*−*31*). Its capsid maturation process is similar to those of other dsDNA phages, as well as herpesviruses and adenoviruses (*32*−*36*). Approximately 150−300 copies of scaffolding protein (gp8, 33 kDa) copolymerize with 420 copies of coat protein (gp5, 47 kDa) and other minor proteins to form a precursor capsid, called the procapsid (*37*−*40*). In vivo, this unexpanded procapsid is the substrate for DNA packaging (*25, 36*). During the DNA packaging process, scaffolding protein exits from the procapsid intact, and the viral genome is condensed in a headful manner accompanied by procapsid expansion that results in several profound morphologic changes. The procapsid is a closed spherical shell having a 580 Å diameter, while the mature capsid is polyhedral with 603 Å × 590 Å dimensions (*41*). During the expansion process, there is extensive internal mass rearrangement, holes visible in the procapsid at the 5-fold and 6-fold axes close, and the procapsid shell wall thins considerably, which has led investigators to conclude that the protein folding process for P22 coat protein is not complete until the capsid is fully matured (*41*−*44*).

Both temperature-sensitive-folding (*tsf*)[1] and cold-sensitive (*cs*) mutants in the coat protein of P22 have been isolated (*45, 46*). These single amino acid substitutions allow viable phage production at permissive temperatures, usually 30 °C. At elevated temperatures, *tsf* coat proteins fold improperly and form inclusion bodies rather than assembling into procapsids unless rescued by an overproduction of the chaperones GroEL/S (*47, 48*). At lowered temperatures, *cs* mutants exhibit changes in coat protein−scaffolding protein interactions and capsid expansion dynamics (*49*). The *tsf*

† This work was supported by the Patrick and Catherine Weldon Donaghue Foundation Grant 95-001 and by NIH Grant GM53567.

* To whom correspondence should be addressed: Department of Molecular and Cell Biology, University of Connecticut, 75 N. Eagleville Rd., U-125, Storrs, CT 06269-3125. Tel. 860-486-4282; Fax 860-486-4331; E-mail teschke@uconnvm.uconn.edu.

[1] Abbreviations: WT, wild type; *tsf*, temperature-sensitive-folding; shells, procapsids with the scaffolding protein removed; GuHCl, guanidine hydrochloride; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride.

10.1021/bi991956t CCC: $19.00
Published on Web 01/12/2000

mutations were considered essentially silent if the mutant phage was grown at permissive temperatures, as virion particles produced remained stable when raised to restrictive temperatures (*50*). Assembly and aggregation reactions monitored in vitro mimic those observed in vivo (*29*). Therefore, these *tsf* and *cs* mutations, together with amber mutations that block phage growth at various specific steps in the maturation process, make P22 coat protein a particularly useful model for the study of the folding and assembly of large multimeric proteins.

The folded conformation of the monomers of the *tsf* coat protein mutants displays changes in both secondary and tertiary structures, as well as an increase in hydrophobicity (*29*). Since enhanced stabilization of the folded conformation occurs during assembly and capsid maturation, we investigated the effect of the *tsf* amino acid substitutions on the assembled state. We selected several *tsf* coat protein mutants in which the single amino acid substitutions are scattered throughout the sequence. Without exception, we found that a single amino acid substitution changes the conformation of the subunit in the procapsid and expanded capsid, even when grown at permissive temperatures.

MATERIALS AND METHODS

Chemicals. Ultrapure guanidine hydrochloride (GuHCl) and urea were purchased from ICN Biomedicals, Inc. Trypsin attached to beaded agarose was from Sigma. HGT Seakem agarose was from American Bioanalytical. All other chemicals were reagent grade from common sources.

Buffer. All experiments described were carried out in 50 mM Tris base, 25 mM NaCl, and 2 mM EDTA, adjusted to pH 7.6 with HCl.

Purification of Procapsids. Procapsids were prepared as described previously (*40*, *42*, *49*). Briefly, *S. typhimurium* was grown at 28 °C to 4×10^8 cells/mL and infected with bacteriophage P22, carrying either WT or a temperature-sensitive mutation in gene 5 and amber mutations in gene 2 or 3 and 13 that block DNA packaging and cell lysis. The *tsf* mutants used substituted glutamine for tryptophan at position 48 (W48Q), valine for alanine at position 108 (A108V), glycine for aspartic acid at position 174 (D174G), asparagine for aspartic acid at position 174 (D174N), aspartic acid for glycine at position 232 (G232D), aspartic acid for glycine at position 282 (G282D), glycine for aspartic acid at position 302 (D302G), and histidine for tyrosine at position 411 (Y411H).

The infected cells were grown for 4.5 h and then pelleted by centrifugation. After suspension in a small amount of buffer, the cells were lysed by one freeze/thaw cycle. The thawed lysate was brought to a final concentration of 0.1 M phenylmethanesulfonyl fluoride (PMSF) and treated with RNase and DNase, and the procapsids were pelleted in a Beckman L7−65 with a Ti60 rotor at 45 000 rpm for 35 min. The procapsid pellets were shaken overnight at 4 °C in a small amount of buffer with PMSF and purified over a Sephacryl S1000 column (Pharmacia LKB). The procapsids were pelleted and suspended again in buffer and stored at 4 °C. Shells were prepared by repeated treatment with buffer containing PMSF and 0.5 M GuHCl, which extracts scaffolding protein while leaving the empty shells intact. After the third extraction, the empty shells were stored in buffer at 4 °C.

Heat Expansion of Shells. Shells at 1 mg/mL were placed in a preheated tube in a water bath at temperatures ranging from 48 to 73 °C. The temperature was controlled by a Fisher Isotemp immersion circulator, model 7305, which has a temperature stability of ±0.05 °C. Aliquots were removed at timed intervals and placed in a tube held on ice containing agarose gel sample buffer (40 mM Tris base, 1 mM EDTA, and 20% sucrose, pH 8.3 with acetic acid). Approximately 6 μg of protein was loaded in each lane of a 1.2% Seakem HGT agarose gel made with the same buffer except without any sucrose and run at a constant 50 V for 3.5 h at room temperature. The gels were stained for 1 h with 10% acetic acid containing 0.03% Coomassie brilliant blue R-250 and 0.02% Coomassie brilliant blue G-250 and then destained over 2−3 days with a solution of 10% acetic acid and 10% isopropyl alcohol. The bands were quantified with a Kodak digital science electrophoresis documentation and analysis system. The kinetics of some expansion reactions were analyzed by use of a first-order rate equation with Kaleidograph 3.0 software from Abelbeck Software.

Scaffolding Protein Extraction. Procapsids at 50 μg/mL were incubated in buffered urea with concentrations ranging from 0.0 to 2.5 M at 20 °C for 18 h. An increase in scaffolding protein extraction corresponds to a decrease in light scattering (*49*). Light scattering was monitored on an SLM Aminco-Bowman 2 spectrofluorometer with excitation and emission wavelengths set to 500 nm, the band-passes set to 4 nm, and the photomultiplier voltage set to 500 V. The fraction of scaffolding retained in the procapsid at the end of incubation is described by the formula $(X_{obs} - X_{extract})/(X_n - X_{extract})$, where X_{obs} is the observed light scattering, $X_{extract}$ is the light scattered by fully extracted empty shells, and X_n is the light scattered by untreated procapsids (*49*)

Proteolysis of Expanded and Unexpanded Shells. Agarose beads with attached trypsin were washed with cold buffer three times, brought to a 1:1 slurry of beads to buffer, and held on ice until use. Shells were brought to 1 mg/mL and either held on ice or heated for 15 min at the lowest temperature necessary to induce 100% expansion and then held on ice. Expansion was verified on an agarose gel. Aliquots of each mutant shell, expanded and unexpanded, were placed in a microcentrifuge tube and treated with a protease slurry in a ratio of 10:1. The tubes were placed on a Nutator for 4 h at room temperature. At the end of the incubation period, the tubes were pulsed in a microfuge for approximately 5 s to pellet the protease beads, and an aliquot was removed from the supernatant and immediately heated at 95 °C for 5 min in SDS sample buffer. The samples were run on Tris−Tricine SDS acrylamide gels, pH 8.45. The separating and the stacking gel were 16.5% and 4% total acrylamide, respectively, both with 3% cross-linker (*51*). Each gel was run at 30 mA (constant) for 18 h at 4 °C and then stained with Coomassie Blue.

Circular Dichroism. Samples were prepared at a concentration of 0.2 mg/mL of each shell. Half of each sample was heated for 15 min and expansion was confirmed on an agarose gel as described above. The spectra, an average of three scans, were collected in a Jasco J-715 spectropolarimeter thermostated at 20 °C with a Jasco PTC-348W1. The system was set to scan from 250 to 200 nm at 50 nm/min, with a response time of 8 s, a bandwidth of 2 nm, a step resolution of 0.5 nm, and a path cell length of 2 mm.

Differential Scanning Calorimetry. A 900 μL aliquot of a 1 mg/mL solution of shells of each shell stock was placed in an ampule in a CSC Model 4100 multicell differential scanning calorimeter. The samples were scanned from room temperature to 98 °C at a 10.0 deg/h scan rate. Data were analyzed with PeakFit software from SPSS, Inc. Enthalpies of denaturation were calculated from the sum of the areas of the endothermic denaturation and the concomitant exothermic aggregation curves.

Urea Denaturation of Shells. Shells at a final concentration of 0.5 mg/mL were incubated in buffered urea with final concentrations ranging from 0.0 to 8.0 M urea at 20 °C for 18 h. Each sample was run on an agarose gel as described above.

RESULTS

P22 bacteriophage carrying *tsf* amino acid substitutions in the coat protein form viable progeny when infections are carried out at permissive temperatures (*47*, *50*). At elevated temperatures, procapsids are unable to assemble and coat protein is instead shunted to off-pathway reactions leading to aggregation and inclusion body formation (*47*, *50*). These mutants were originally characterized as folding, not assembly, mutants (*42*, *45*). On the basis of their phenotype, it does not immediately follow that capsids made of *tsf* coat protein should differ in any way from capsids composed of WT coat protein, as long as they have successfully assembled into procapsids at permissive temperatures, rather than aggregated into inclusion bodies. Many proteins with only single amino acid substitutions are phenotypically indistinguishable from WT proteins (*52*, *53*). However, *tsf* and WT coat protein monomers are not identical even when folded at permissive temperatures (*29*); perhaps through interactions between subunits the oligomeric structures can correct any conformational defects to allow maturation from procapsids to viable phage. To investigate the extent of the effects of the single amino acid substitutions on *tsf* coat proteins, procapsids containing either WT or *tsf* coat protein were grown at permissive temperatures in vivo, harvested, processed, and probed in vitro using a variety of biophysical and biochemical techniques.

The Process of Expansion Is Altered by Tsf Amino Acid Substitutions. If WT and mutant coat proteins are able to assemble into procapsids and then eventually form infectious particles in vivo, the procapsids must be able to undergo the expansion process (*38*, *54*). To determine if the coat protein monomers bearing *tsf* amino acid substitutions caused changes in the procapsid expansion process, the expansion rates were monitored when shells were treated with heat (Figure 1) (*49*, *55*). The heat-treated samples were run on agarose gels, the bands in each lane were quantified, and the percentage of expanded shells was calculated.

The kinetics of the expansion process for WT, A108V, and G282D shells were readily described by first-order kinetics (Figure 2A), while the expansion kinetics of the other six *tsf* mutants were more complicated (Figure 2B). For W48Q, D174G, D174N, G232D, D302G, and Y411H shells, a lag phase was apparent at lower temperatures (Figure 2B). If the incubation temperature was raised, eventually enough thermal energy was present to overcome the energy barrier, and the lag phase was no longer observed (Figure 2B). The

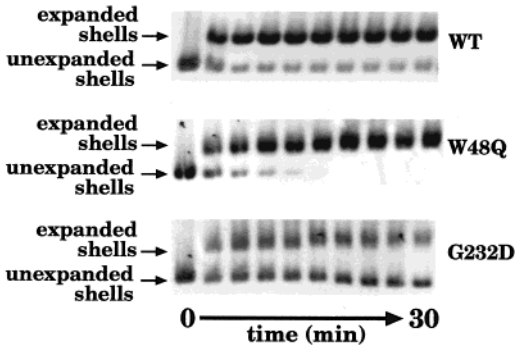

FIGURE 1: Heat expansion of WT and *tsf* shells. Shells at 1 mg/mL were incubated for 2, 3, 4, 5, 10, 15, 20, 25, and 30 min. In the examples shown, the incubation temperature was 63 °C. The samples were run on 1.2% HGT agarose gels as described under Materials and Methods. The percent of shells expanded was calculated for each time point by dividing the intensity of the expanded shell band by the sum of the intensities of the expanded and unexpanded shell bands.

subpopulation of procapsids that did not expand at the lower temperature was easily expanded if the sample was shifted to a higher temperature (data not shown). W48Q shells have previously been reported to be thermolabile and incapable of heat-induced expansion. We attempted to reproduce the experiments as described but were unable to replicate the results (*56*, *57*). The gene that codes for coat protein was sequenced from a phage stock derived from the few residual phage in our W48Q procapsid preparation, and the mutant used here was indeed W48Q. We remain uncertain of the reason for the differences between our results and those of de Sousa et al. (*57*).

To quantify the extent to which the amino acid substitutions were exerting effects on the expansion process by generating an Arrhenius plot from which the energies of activation could be calculated, the first-order rates of expansion were needed (*57*). It was not possible to mathematically fit six of the *tsf* mutants to first-order rate equations (Figure 2b), and so they could not be considered for such analysis. However, the validity of the application of Arrhenius behavior to those shells that were fit well by first-order rate equations must be carefully examined. Since only a portion of the population of WT, A108V, or G282D shells expanded even after extended incubation periods at lower temperatures, a heterogeneity was clearly present in the samples which indicated that the shells were in an ensemble of states, and thus classic Arrhenius behavior could not apply. Consequently, we have decided that our analysis must remain qualitative, rather than quantitative. The heterogeneity of populations seen during the expansion of the shells suggests that not all coat proteins reached the same conformation even given the same conditions for folding and assembly. Since more than one conformation was acquired during folding, this indicated a high degree of conformational flexibility in coat protein. When the rates of expansion of the mutant shells were compared to WT shells, it was clear that each mutant displayed expansion behavior that was distinct from that of WT shells. For instance, if we compared the temperature range required to expand the shells, D174G, D174N, and D302G showed a narrow range of temperature (~3−6 °C) where the transition from 20% of the capsid population expanded to 100% expanded occurred as compared to WT shells. A108V had a 17 °C range for expansion,

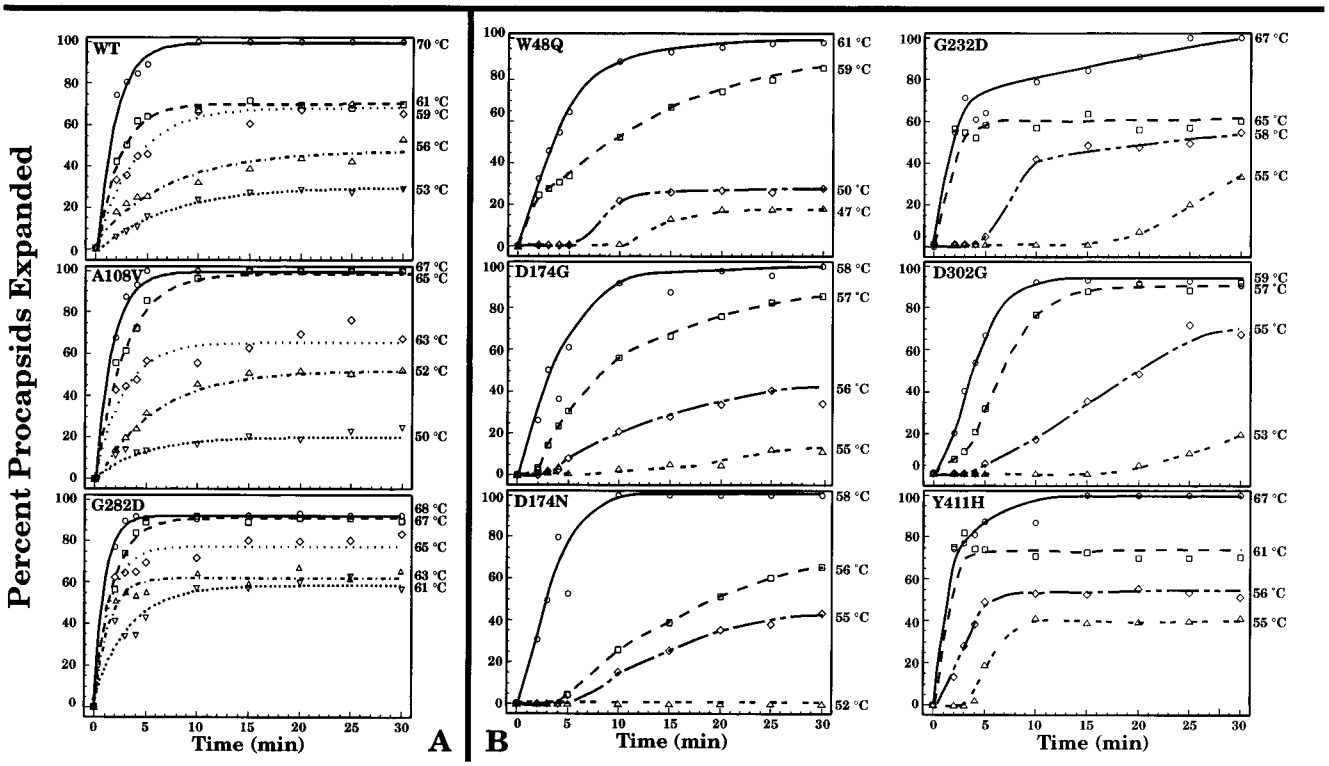

FIGURE 2: Kinetics of expansion of WT and *tsf* shells. Heat expansion data, such as those shown in Figure 1, were used to determine the percent of expanded shells at each time point for a given temperature. The temperatures, which are indicated on the right side of the graphs, were chosen to best illustrate the various kinetics observed. In panel A, the lines show the fit of the data to first-order kinetics. In panel B, the lines were drawn to aid the eye and are not meant to represent the fit of the data to any model.

which was equal to WT; however, the entire range was shifted down 3 °C, from 50−67 °C as opposed to 53−70 °C for WT shells. Conversely, G282D had both an extended and a higher temperature range of 20−100% expansion from 53 to 73 °C. Additionally, the presence of a lag phase in the kinetics of expansion of some mutant shells, but not in others, suggests that that rate-limiting step in the expansion of those mutants changed, indicating that there must be intermediates in the expansion process. Taken together, these data indicate that the conformation of *tsf* coat protein remained altered from that of WT even when assembled into procapsids.

Scaffolding Protein Extraction Is Modified in Procapsids with Tsf Amino Acid Substitutions. In order for expansion to proceed, scaffolding protein must first be released (*38, 46, 55*). Observing that the expansion processes of the *tsf* mutant shells differed from WT, we suspected coat protein−coat protein interactions were somehow altered. To determine if *tsf* coat protein interactions with WT scaffolding protein were also altered, scaffolding protein release from the procapsids was monitored by the decrease in light scattering caused by exit of scaffolding protein as a result of incubation in urea of varied concentrations (*49*). While capsids have been shown to expand with denaturant (*44, 58*), little to no expansion occurs from 0.0 to 2.2 M urea, which is the range of concentrations where these experiments were carried out (*49*). Even when observing W48Q procapsids, where some expansion does occur prior to 2.2 M urea (data not shown), no increase in light scattering due to expansion was observed (Figure 3). This result would indicate that when scaffolding protein is present, the signal from scaffolding protein exiting the procapsid overwhelms any contribution to the light scattering from small populations of expanded procapsids.

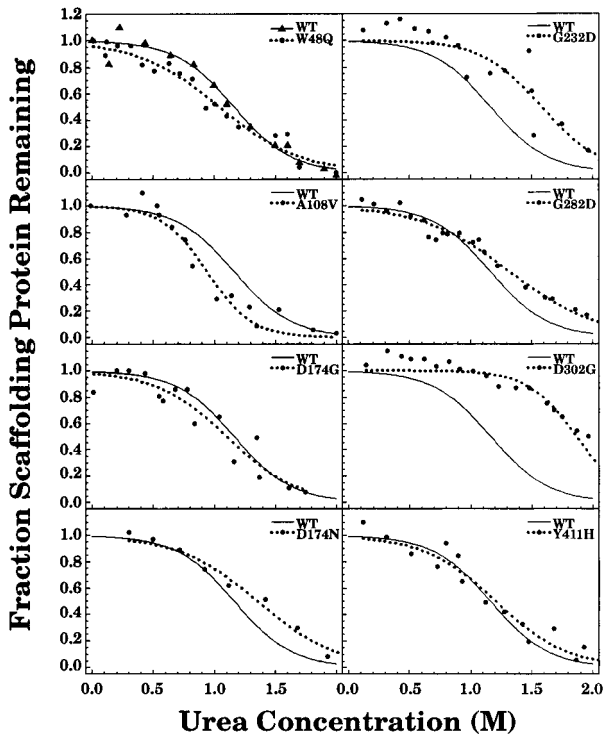

FIGURE 3: Release of scaffolding from WT and *tsf* procapsids. WT (▲, solid lines) and *tsf* (●, dashed lines) procapsids at 50 μg/mL were incubated in urea. The fraction of scaffolding remaining within the procapsids at each urea concentration was determined by light scattering as described under Materials and Methods. The lines drawn are to aid the eye and are not meant to represent the fit of the data to any model. The curve for the release of scaffolding protein from WT procapsids is shown in each panel for comparison.

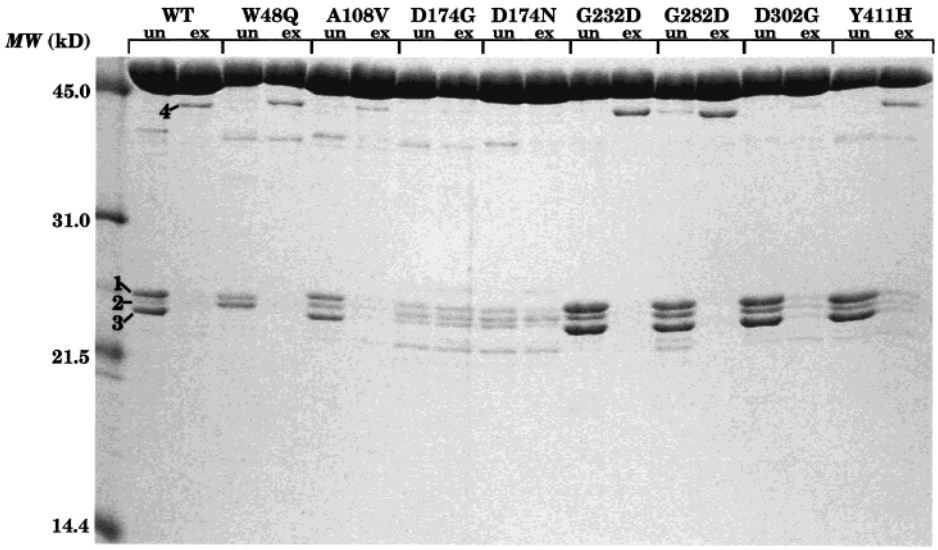

FIGURE 4: Proteolysis of unexpanded and expanded WT and *tsf* shells. Unexpanded (un) and heat-expanded (ex) shells at 1 mg/mL were incubated with trypsin attached to agarose beads for 4 h. The resulting peptides were run on Tris−Tricine 16.5% polyacrylamide gels. The major products of digestion were numbered 1−3 in the WT unexpanded sample lane and 4 in the WT expanded sample lane.

Table 1: Release of Scaffolding Protein from Procapsids

shell	$c_{1/2}$[a] (M)	shell	$c_{1/2}$[a] (M)
WT	1.16	G232D	1.57
W48Q	1.04	G282D	1.31
A108V	0.94	D302G	1.87
D174G	1.09	Y411H	1.21
D174N	1.35		

[a] The $c_{1/2}$ is the concentration of urea required to release 50% of the scaffolding protein from the procapsid.

Three of the *tsf* mutant procapsids required a lower urea concentration for release of 50% of their scaffolding protein, while five *tsf* mutants required a higher concentration as compared to WT procapsids (Table 1). Since W48Q, A108V, and D174G required less than 1.10 M urea to reach the midpoint of extraction, scaffolding protein must bind less tightly to the interior of these mutant procapsids than to WT procapsids, which required 1.16 M urea. D174N, G232D, G282D, and Y411H all reached the midpoint of scaffolding release at a urea concentration from 1.04 to 1.43 times that of WT. D174N is particularly interesting because even though it is at the same location as D174G, D174N did not bind scaffolding less tightly than WT. The changes in conformation caused by the glycine or asparagine substitution at position 174, which is likely to be located at the surface of the capsid (*59*), must be propagated throughout the mutant coat protein for the capsid interior to be affected, and the asparagine must cause a different conformational change than the glycine substitution. The extraction of scaffolding protein from D302G was markedly different from WT, as well as all of the other seven *tsf* mutants, because not only did it require 1.87 M urea to release 50% of the scaffolding protein, 1.61 times as much as WT, but also D302G was much closer than any other procapsid to total dissociation prior to the release of 50% of the scaffolding protein. This may indicate that scaffolding protein was tightly bound to D302G coat protein at the inner procapsid surface, or it may indicate that a change in conformation of the coat protein caused a reduction in the diameter of the holes located at the center of the hexameric capsomeres. What is clear is that since all of these experiments were performed with WT scaffolding protein, every perturbation in the release of scaffolding protein must be attributed to changes in the coat protein.

Exteriors of WT and Tsf Capsids Are Dissimilar before and after Expansion. One reason for altered scaffolding protein release from procapsids would be a change in the conformation of the coat protein in the unexpanded procapsids. This change could alter the interior surface of the procapsid by eliminating or adding additional binding sites for scaffolding protein, or it could block the exit holes present in the procapsids at the quasi-6-fold positions (*41*). To probe the extent of changes on the exterior surface, limited proteolysis with trypsin attached to agarose beads was performed on unexpanded and expanded shells. The proteolytic pattern of all of the shells showed many similarities, but there were no shells composed of *tsf* coat protein whose proteolytic patterns were identical to that of WT shells (Figure 4). For instance, band 2 in the digestion of G232D, G282D, D302G, and Y411H shells was much darker in comparison to band 2 of WT shells, and band 3 was entirely missing from the proteolysis pattern of W48Q shells. All of the peptide bands seen in lanes containing D174G and D174N shells were much less intense than the bands seen in the lanes with WT protein. In addition, all of the mutant shells except W48Q contained at least one additional band that was not present in the digestion of WT shells.

Once expanded, WT, shells have fewer sites available for proteolytic digestion (*55*, *59*), and most of the mutants also followed this pattern (Figure 4). However, the digestion of expanded G232D and G282D was somewhat different as band 4 in the digestion pattern of these mutants was more intense than band 4 of expanded WT shells. Conversely, band 4 was less intense in the digest of expanded D302G shells than band 4 in the digest of expanded WT shells and was entirely absent from the digests of expanded D174G and D174N shells. D174N and D174G were radically different from WT shells; there was no noticeable change in proteolytic pattern from unexpanded to expanded D174N and D174G shells. Thus, the conformation of the *tsf* coat proteins remained different from that of the WT conformation even after expansion.

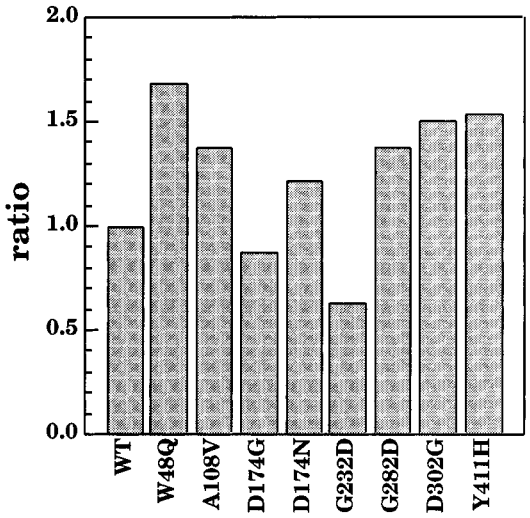

FIGURE 5: Secondary structure differences probed with circular dichroism. The ratio is equal to $[(I_{222}\ tsf\ \text{shells}_{un} - I_{222}\ tsf\ \text{shells}_{ex})/(I_{209}\ tsf\ \text{shells}_{un} - I_{209}\ tsf\ \text{shells}_{ex})]/[(I_{222}\ \text{WT shells}_{un} - I_{222}\ \text{WT shells}_{ex})/(I_{209}\ \text{WT shells}_{un} - I_{209}\ \text{WT shells}_{ex})]$, where *I* denotes the intensity of the signal at the wavelength indicated, un denotes unexpanded shells, and ex denotes expanded shells. Thus, numbers greater than 1 indicate that the mutant had a greater change in signal at 222 nm with respect to 209 nm from unexpanded to expanded than the WT shells. Numbers less than 1 indicate that the change for the *tsf* mutant was less than for that of WT.

Secondary Structure Change Caused by Expansion Differs in Shells with Tsf Amino Acid Substitutions. The differences observed between *tsf* and WT capsid lattices during heat expansion, scaffolding extraction, and proteolytic cleavage could be due to changes in secondary structure. Therefore, the secondary structure was investigated by circular dichroism. Spectra of unexpanded and expanded WT and *tsf* shells were taken and difference spectra were generated (data not shown). Changes were seen in secondary structure, as expected (*60, 61*). To quantify the extent of the structural changes, the ratio of the magnitude of the signal at 222 nm in relation to the signal at 209 nm for the difference spectra of each of the shells was calculated and compared to the same ratio for WT shells (Figure 5). It was clear that the degree of change in secondary structure was not identical for all of the shells examined. Again, these results indicated that even expansion does not correct changes in the conformation of coat protein due to the *tsf* amino acid substitution.

Stability of the Shells Is Changed by Tsf Substitutions. Because changes were observed in protein−protein interactions, protease resistance, and secondary structure, we suspected the stabilities of the shells made of *tsf* coat protein would differ from the stability of WT shells. To probe the differences between the various shells, WT and *tsf* mutant shells were observed by DSC at 10.0 deg/h from 15 to 98 °C. The main transition seen corresponds to capsid dissociation and irreversible denaturation (*55*). Prior to the main transition, the shells have expanded. The scan rate was reduced 10-fold compared to Galisteo and King (*55*) in order to shift the T_m low enough to ensure the instrument could return to baseline after the final transition because we were using a CSC Model 4100 multicell differential scanning calorimeter, which does not have a pressurized chamber (*55*). The much reduced scan rate uncovered a transition not visible at the higher scan rate. This transition, which was a sharp exotherm indicative of aggregation (*62*), occurred concomitantly with the endothermic denaturation transition. To ascertain whether the three peaks were in fact two concomitant processes or three separate processes, these scans were repeated but halted prior to the exotherm, cooled, and then heated beyond the exotherm. If there were three processes involved, then one peak would be visible in the first cycle and the other two in the second cycle (*63*). However, this was not the case. The first endotherm was present in the first cycle, and the exotherm was present in the second cycle (data not shown), indicating that the second endotherm seen was the end of a single endothermic process interrupted by the exothermic process.

During the exothermic aggregation process none of the mutants precipitated identically to WT (Figure 6). For example, D174G and D174N were almost completely denatured prior to aggregation as seen by the minimal remaining endotherm following the exotherm. Both transitions occurred over a smaller temperature range for A108V as compared to WT. For G282D, the denaturing process initially had a broader transition than WT, followed by a broader exotherm during which aggregation occurred, and then completed with the second half of the endotherm similar to WT shells. Melting temperatures of the expanded shells of several *tsf* mutants were similar to that of expanded WT shells at 82.2 °C (Figure 6). The melting temperatures for W48Q, A108V, G282D, and D302G expanded shells varied from 77.8 to 82.8 °C, while D174G, D174N, G232D, and Y411H shells were noticeably less stable (Table 2). The area of the exotherm was subtracted from the sum of the areas of the endotherm to calculate the change in enthalpy for the main transition of each of the expanded shells (Table 2). Here again, none of the expanded shells containing *tsf* coat protein were identical to WT, although A108V and D174G were quite similar. Y411H shells exhibited the greatest increase in enthalpy compared to WT shells, while G232D shells had the largest decrease.

As another method to observe changes in shell stability, WT and *tsf* shells were treated with various concentrations of urea for 18 h. Capsid dissociation could be followed via changes in light scattering, but a fraction of shells treated with urea expand prior to dissociation, as visualized on agarose gels (data not shown). Since the light scattering signal of a sample is the average of the scattering of all of the molecules present, an endpoint analysis based on agarose gels was chosen to avoid the errors that would be introduced in the light scattering of samples containing multiple species of different diameters. As seen in Figure 7, 6.0 M urea was required to completely denature WT shells. When subjected to chemical denaturant, no *tsf* shell was as stable as that composed of WT coat protein. Of the mutants, shells of A108V were the most resistant to denaturation, which occurred at 5.25 M urea, and the rest of the mutant shells were completely dissociated at concentrations lower than 5.0 M urea. D302G shells were dramatically different from WT shells, dissociating entirely with only 3.0 M urea. Thus, each mutant shell showed changes in stability as monitored by chemical denaturation and DSC.

DISCUSSION

Proteins reach the correct three-dimensional structure via a folding pathway directed by the amino acid sequence (*1*).

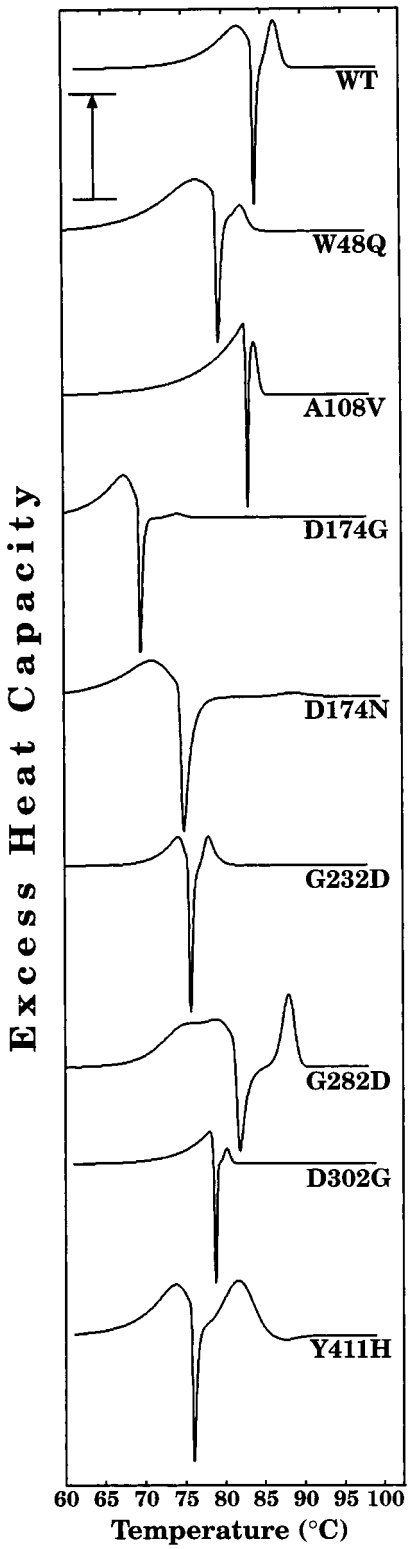

FIGURE 6: Thermograms of WT and *tsf* shells. Protein concentration was 1 mg/mL. The scan rate of the calorimeter was 10.0 K/h, and the baseline was corrected for instrumental baseline and chemical baseline using PeakFit. The data were normalized for protein concentration. The bar corresponds to 200 kJ/K·mol, and the arrow shows the direction of heat absorption.

Studies have shown that proteins are remarkably accommodating to amino acid substitutions, particularly those that are on the surface of the native protein (*3, 52, 64*). In some proteins, however, a single amino acid substitution is sufficient to seriously impair or even totally undermine the

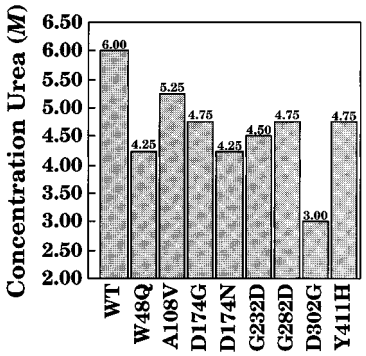

FIGURE 7: Urea denaturation of WT and *tsf* shells. WT and *tsf* shells at 0.5 mg/mL were incubated overnight in buffered urea. The samples were run on an agarose gel as described under Materials and Methods. The number above each bar indicates the lowest concentration of urea at which total dissociation of shells occurred.

Table 2: Differential Scanning Calorimetry of Shells

shell	T_m (°C)	ΔH (kJ·mol^{-1})	shell	T_m (°C)	ΔH (kJ·mol^{-1})
WT	82.5	1651	G232D	75.7	559
W48Q	79.4	2747	G282D	82.5	2066
A108V	82.5	1854	D302G	77.8	1239
D174G	69.5	1501	Y411H	74.9	3080
D174N	67.4	1060			

folding, structure, and function of the protein (*13*−*15, 17, 29, 65*). Further, many proteins require additional modification such as oligomerization to reach the native conformation. Association of subunits in multimeric proteins may facilitate or even be required to attain the native conformation (*19*−*21, 66, 67*). In the coat protein of P22 bacteriophage, 18 single amino acid substitutions have been identified that cause defects in folding (*45, 50*). Here we have investigated the extent of the influence of *tsf* single amino acid substitutions on the folding process through to capsid maturation.

Criteria by Which the Tsf Mutants Were Chosen. In preliminary experiments, we found that each of the 18 *tsf* amino acid substitutions caused differences as compared to WT shells, regardless of the property of the amino acid side chain or the location of the substitution in the primary amino acid sequence. Thus, we needed some criteria to limit the number of mutants with which we worked. We selected amino acids substitutions throughout the linear sequence so that the probability of all the substitutions being located in proximity to one another in the folded coat protein would be minimized. Five of the *tsf* mutants we chose had substitutions that involved a charge change, which allowed confirmation that the *tsf* amino acid substitutions were distributed throughout the three-dimensional structure since charge changes in the protein core or on the interior face of the capsid are not observable on a native agarose gel, while charge changes on the shell surface are seen as shifts in mobility (*68*). D174N and D174G, which each lose a negatively charged residue, and G232D, which gains one, exhibited a reduction and an increase in mobility, respectively (data not shown), which indicated that amino acids 174 and 232 were situated on the outside surface of the shells. G282D and D302G, which also each have a charge change, showed a mobility identical to WT shells and to *tsf* mutants without charge changes, and so amino acids 282 and 302 must be

located either in the interior of the capsid, in the interior of the coat protein monomer, or in a site of coat protein−coat protein interaction. As the detailed structure of coat protein is not known, the spatial placement of the amino acid substitutions in the remaining *tsf* mutants cannot be determined. The varied locations of the *tsf* amino acids are in contrast to P22 tailspike protein, where virtually all of the *tsf* residues reside in the central domain, a large parallel β-helix (*22*, *24*).

In addition to having the *tsf* substitutions scattered throughout the linear sequence, the mutants used in this study were chosen from parents of various categories of second-site suppressors that we have identified (*69*). These suppressors are single amino acid substitutions that suppress the folding defect in the parent *tsf* coat protein mutants. Global suppressors, amino acid substitutions at a second site that correct the defect of several *tsf* mutants, have been identified for A108V, D174G, D174N, and G282D. Y411H has a second-site suppressor that is likely to be involved in the local structure, D302G has a same-site suppressor, and W48Q reverts consistently to the WT sequence, having no known second-site suppressor. G232D has been shown to have a suppressor mutation that is not identifiable anywhere in the coat protein sequence and is thought to be extragenic. Characterization of the mutants chosen here, which also carry a second-site suppressor, is currently underway (Aramli and Teschke, unpublished results). Therefore, by use of several criteria, a broad spectrum of *tsf* mutants was chosen to develop a comprehensive picture of the extent of the effects of single amino acid substitutions on protein folding and oligomeric assembly.

Folding Defects Are Not Alleviated by Assembly. In vivo, *tsf* coat proteins are assembly-competent and can form viable phage at permissive temperatures, but at elevated temperatures, destabilized folding intermediates form inclusion bodies (*50*). While WT coat protein does not employ the molecular chaperone complex GroEL/S to fold properly, *tsf* coat proteins utilize GroEL/S to fold into an assembly-competent conformation, rather than forming aggregates (*47*, *48*). However, the rate of assembly of *tsf* mutant procapsids is reduced when compared to WT procapsids (*47*). In vitro, conditions have been found where *tsf* mutants, which fold into conformations different from that of WT coat protein, aggregate and assemble, mimicking the in vivo phenotype (*29*). If the successful assembly of procapsids forced coat protein to adopt the correct native WT conformation, either through interactions with scaffolding protein or through coat protein−coat protein interactions, then as soon as procapsids were formed, defects in the coat protein conformation should no longer be apparent. However, this was not the case. For example, scaffolding protein interacted differently with *tsf* mutant coat proteins as compared to WT coat protein. Since D174G, D174N, and G232D are mutants with *tsf* amino acid substitutions on the outside surface of the procapsid, there cannot be a direct correlation between the charge change and a change in interaction with scaffolding protein. Therefore, a more global conformational change must be propagated by local changes in the polypeptide fold. As another example, procapsids comprising D302G needed to be incubated in a higher urea concentration than WT procapsids in order to release scaffolding, as though, perhaps, this amino acid substitution is on the interior of the capsid and more tightly bound to the scaffolding than WT coat protein. However, D302G shells also dissociate more readily than WT shells, whether treated with a chemical denaturant or with heat, which implicates monomer−monomer interactions as the cause for D302G having a *tsf* phenotype. Again, the conclusion that a single amino acid substitution affects only its local environment would be erroneous. These differences in the coat protein conformation are readily apparent in the proteolytic digestion patterns, as well. Some of the *tsf* mutant shells have different sites available for cleavage than WT shells and so are not identical even after successful assembly.

The Expansion Process Does Not Correct Folding Defects. If the assembly of the procapsids had locked the *tsf* mutants into WT conformation, then there should not have been differences in the expansion kinetics of the *tsf* mutants when compared to the WT shells. Yet five of the single amino acid substitutions caused a prominent change in the rate-limiting step of the expansion process as evidenced by the lag phase apparent at lower temperatures. The rate-limiting step was changed for mutants with both buried and external *tsf* amino acid substitutions and for mutants with *tsf* amino acid substitutions that added or removed a negative charge. In these expansion experiments, the reorganization caused by intramolecular movements that occur during expansion were probed, as the coat protein does not need to be cleaved or to form covalent bonds with its neighboring monomers in order for expansion to proceed (*44*). Our results suggest that the single amino acid substitutions continue to perturb the conformation of the monomers during the expansion process, as well as during folding and assembly. Additionally, if the *tsf* coat protein mutants had been constrained in the native WT conformation after expansion occurred, then differences between *tsf* mutant and WT shells would not have been visible in the secondary structure, the trypsin cleavage sites, or in stability of the expanded shells, suggesting significant plasticity remains present in the monomers after expansion. Hence, there appear to be altered conformations of the folding intermediates of coat protein and also changes in the final conformation and stability achieved by the *tsf* monomers. Coat proteins with the *tsf* amino acid substitutions are able to form monomers that are capable of producing viable phage at permissive temperatures without identically matching WT folding. Still, regardless of the properties of the side chain or its location in the primary sequence, the *tsf* single amino acid substitutions altered the conformation of coat protein throughout the folding process to the mature capsid.

Comparison of Effects of Amino Acid Substitutions in Folding and Assembly of Several Proteins. We have shown that plasticity in coat protein persists throughout folding and assembly. Such malleability is necessary for the monomers to attain the various conformations needed throughout the phage life cycle and has the added benefit of conferring tolerance to at least some degree of conformational differences. This not unlike *Vibrio harveyi* luciferase. When refolded in nonnative conditions, such as separate monomers, each monomer retains enough similarity to the native conformation to exhibit bioluminescence, albeit with greatly reduced intensity. Both the luciferase α and β subunits and the *tsf* coat protein subunits are altered from the native conformation, yet similar and still at least partially active. Akin to coat protein assembly, luciferase mutations affect

subunit association kinetics (*29, 70*). In terms of stability, coat protein does not resemble luciferase mutants, which have stabilities comparable to WT luciferase (*70, 71*).

Tsf mutants have also been identified in P22 tailspike protein (*72, 73*). Like *tsf* coat protein mutants, tailspike protein mutants were isolated as exclusively folding mutants since early studies showed no influence on the stability of the native protein (*23*). Newer, more detailed studies disclosed an increase in the rate of unfolding at a higher temperature and that the transition midpoints of urea denaturation curves were shifted to significantly lower urea concentrations, revealing the tailspike mutants have a reduced stability as compared to the WT tailspike protein (*22, 74*). For two truncated versions of tailspike protein, spectroscopic data show *tsf* tailspike protein mutants retain nativelike conformation, and binding assays show reasonable activity also present (*75, 76*). However, there are thermolabile folding intermediates generated by the *tsf* amino acid substitutions that are responsible for kinetic partitioning between productive and nonproductive pathways.

Interleukin 1β also partitions between productive folding and inclusion bodies because of an aggregation-prone kinetic folding intermediate (*77*). Unlike coat protein, interleukin 1β need not assemble as it is a monomeric protein. But similarly to coat protein *tsf* mutants, D174N and D174G, interleukin 1β has multiple aggregation-prone mutants with an amino acid substitution at the same site, Lys97, that confer various phenotypes. An arginine substitution does not stabilize the native protein, but it does reduce inclusion body formation, while a valine substituted at position 97 actually increases inclusion body formation even though it stabilizes the native monomer (*78, 79*). Likewise, the coat protein mutants D174G and D174N differ in some respects. We found that the thermal denaturation and stability to denaturant of these mutants were similar, though not identical. However, Gordon and King (*45*) showed that the D174N mutation is a dominant phenotype, while D174G is not.

In addition to interleukin 1β, Wetzel et al. (*80*) also worked with γ-interferon. Using randomized cassette mutagenesis, they produced mutants that were prone to inclusion body formation. Also, the stability of the mutants to intracellular proteolysis was altered from that of WT γ-interferon. Surprisingly, two regions were identified that yielded mutants which routinely increased folding efficiency (*80*). Alber et al., (*81*) identified 25 different single amino acid mutations in the lysozyme of bacteriophage T4 that presented a phenotype of temperature-sensitivity. Mutations were induced in a significant fraction of the lysozyme gene, and like P22 *tsf* coat protein mutants, no simple pattern in the properties of the amino acid substitutions that conferred the temperature-sensitive phenotype was identified. Additionally, many of the substitutions affected the stability of lysozyme, as is the case in coat protein (*82, 83*). Unlike P22 *tsf* mutants, the T4 lysozyme mutations all occurred at sites with low solvent accessibility in the folded protein (*81*). Thus, generally substitutions that cause a temperature-sensitive folding phenotype are likely to alter both the stability and the folded conformation of proteins. Since *tsf* substitutions can switch a protein from productive to nonproductive pathways by a simple increase in temperature, they are a powerful tool in elucidating folding and assembly pathways.

Here we have shown that the *tsf* amino acid substitutions in P22 coat protein do not perturb only the folding process, as their effects are not mitigated by capsid assembly or subsequent expansion. Despite the property of the side chain of the *tsf* amino acid substitution or its placement in the linear and subsequent tertiary structure, the *tsf* mutant shells differ from the WT shells when observed with a variety of probes. Still, *tsf* coat protein monomers, despite having altered conformation from WT monomers, are able to assemble and expand, demonstrating a remarkable tolerance and flexibility.

ACKNOWLEDGMENT

We thank Dr. David G. Rhodes for use of his differential scanning calorimetry instrument, Allison Radwick for assistance with DSC scans, Drs. Philip L. Yeagle and Arlene D. Albert for use of their circular dichroism instrument, and Lili Aramli and Shannon Doyle for helpful comments as this work progressed.

REFERENCES

1. Anfinsen, C. B. (1973) *Science 181*, 223−230.
2. Sinclair, J. F., Ziegler, M. M., and Baldwin, T. O. (1994) *Nat. Struct. Biol. 1*, 320−326.
3. Dill, K. A. (1999) *Protein Sci. 8*, 1166−1180.
4. Socci, N. D., Onuchic, J. N., and Wolynes, P. G. (1998) *Proteins*: *Struct., Funct., Genet. 32*, 136−158.
5. Bryngelson, J. D., Onuchic, J. N., Socci, N. D., and Wolynes, P. G. (1995) *Proteins: Struct., Funct., Genet. 21*, 167−195.
6. Ranson, N. A., White, H., and Saibil, H. R. (1998) *Biochem. J. 333*, 233−242.
7. Marston, F. A. O. (1986) *Biochem. J. 240*, 1−12.
8. Mitraki, A., and King, J. (1989) *Biotechnology 7*, 690−697.
9. Teschke, C. M., and King, J. (1992) *Curr. Opin. Biotechnol. 3*, 468−473.
10. Booth, D. R., Sunde, M., Bellotti, V., Robinson, C. V., Hutchinson, W. L., Fraser, P. E., Hawkins, P. N., Dobson, C. M., Radford, S. E., Blake, C. C. F., and Pepys, M. B. (1997) *Nature 385*, 787−793.
11. Carrell, R. W., Lehmann, H., and Hutchison, H. E. (1966) *Nature 210*, 915−916.
12. Carrell, R. W., Bathurst, I. C., and Brennan, S. D. (1984) in *Molecular variants of proteins: Biosynthesis and clinical relevance* (Campbell, P. N., and Phelps, C. F., Eds.) pp 55−66, Biochemical Society Symposium, London.
13. Dalgleish, R. (1997) *Nucleic Acids Res. 25*, 181−187.
14. Dalgleish, R. (1998) *Nucleic Acids Res. 26*, 256−258.
15. Eaton, W. A., and Hofrichter, J. (1990) *Adv. Protein Chem. 40*, 63−279.
16. Helms, L. R., and Wetzel, R. (1996) *J. Mol. Biol. 257*, 77−86.
17. Ingram, V. M. (1957) *Nature 180*, 326−328.
18. Kelly, J. W. (1997) *Structure 5*, 595−600.
19. Robinson, C. R., Rentzeperis, D., Silva, J. L., and Sauer, R. T. (1997) *J. Mol. Biol. 273*, 692−700.
20. Waddle, J. J., Johnston, T. C., and Baldwin, T. O. (1987) *Biochemistry 26*, 4917−4921.
21. Ziegler, M. M., Goldberg, M. E., Chaffotte, A. F., and Baldwin, T. O. (1993) *J. Biol. Chem. 268*, 10760−10765.
22. Schuler, B., and Seckler, R. (1998) *J. Mol. Biol. 281*, 227−234.
23. Goldenberg, D., and King, J. (1981) *J. Mol. Biol. 145*, 633−651.
24. Haase-Pettingell, C., and King, J. (1997) *J. Mol. Biol. 267*, 88−102.
25. King, J., Botstein, D., Casjens, S., Earnshaw, W., Harrison, S., and Lenk, E. (1976) *Philos. Trans. R. Soc. London B 276*, 37−49.
26. Prevelige, P. E., Jr., King, J., and Silva, J. L. (1994) *Biophys. J. 66*, 1631−1641.

27. Thuman-Commike, P. A., Greene, B., Jokana, J., Prasad, B. V. V., King, J., Prevelige, P. E., Jr., and Chiu, W. (1996) *J. Mol. Biol. 260*, 85−98.
28. Thuman-Commike, P. A., Greene, B., Malinski, J. A., Burbea, M., McGough, A., Chiu, W., and Prevelige, P. E. J. (1999) *Biophys. J. 76*, 3267−3277.
29. Teschke, C. M. (1999) *Biochemistry 38*, 2873−2881.
30. Speed, M. A., Wang, D. I. C., and King, J. (1995) *Protein Sci. 4*, 900−908.
31. Teschke, C. M., and King, J. (1993) *Biochemistry 32*, 10839−10847.
32. Black, L. W., and Showe, M. K. (1983) in *Bacteriophage T4* (Matthews, C. K., Kutter, E. M., Mosig, G., and Berget, P. B., Eds.) pp 219−245, American Society for Microbiology, Washington, DC.
33. Morin, N., and Boulanger, P. (1984) *Virology 136*, 153−167.
34. Casjens, S., and Hendrix, R. W. (1988) in *The Bacteriophages* (Calendar, R., Ed.) pp 15−90, Plenum Press, New York.
35. Homa, F. L., and Brown, J. C. (1997) *Rev. Med. Virol. 7*, 107−122.
36. King, J., and Chiu, W. (1997) in *Structural Biology of Viruses* (Chiu, W., Burnett, R., M., and Garcea, R., Eds.) pp 288−311, Oxford University Press, New York.
37. King, J., Lenk, E. V., and Botstein, D. (1973) *J. Mol. Biol. 80*, 697−731.
38. King, J., and Casjens, S. (1974) *Nature 251*, 112−119.
39. Fuller, M. T., and King, J. (1980) *Biophys. J. 32*, 381−401.
40. Prevelige, P. E., Jr., Thomas, D., and King, J. (1988) *J. Mol. Biol. 202*, 743−757.
41. Prasad, B. V. V., Prevelige, P. E., Jr., Marieta, E., Chen, R. O., Thomas, D., King, J., and Chiu, W. (1993) *J. Mol. Biol. 231*, 65−74.
42. Galisteo, M. L., Gordon, C. L., and King, J. (1995) *J. Biol. Chem. 270*, 16595−16601.
43. Earnshaw, W., and Casjens, S. (1980) *Cell 2l*, 319−331.
44. Earnshaw, W., Casjens, S., and Harrison, S. C. (1976) *J. Mol. Biol. 104*, 387−410.
45. Gordon, C. L., and King, J. (1994) *Genetics 136*, 427−438.
46. Greene, B., and King, J. (1996) *Virology 225*, 82−96.
47. Nakonechny, W. S., and Teschke, C. M. (1998) *J. Biol. Chem. 273*, 27236−27244.
48. Gordon, C. L., Sather, S. K., Casjens, S., and King, J. (1994) *J. Biol. Chem. 269*, 27941−27951.
49. Teschke, C. M., and Fong, D. G. (1996) *Biochemistry 35*, 14831−14840.
50. Gordon, C. L., and King, J. (1993) *J. Biol. Chem. 268*, 9358−9368.
51. Schägger, H., and von Jagow, G. (1987) *Anal. Biochem. 166*, 368−379.
52. Bowie, J. U., Reidhaar-Olson, J. F., Lim, W. A., and Sauer, R. T. (1990) *Science 247*, 1306−1310.
53. Hellinga, H. W., Wynn, R., and Richards, F. M. (1992) *Biochemistry 31*, 11203−11209.
54. Casjens, S. (1979) *J. Mol. Biol. 131*, 1−19.
55. Galisteo, M. L., and King, J. (1993) *Biophys. J. 65*, 227−235.
56. Parker, M. H., and Prevelige, P. E., Jr. (1998) *Virology 250*, 337−349.
57. de Sousa, P. C., Jr., Tuma, R., Prevelige, P. E., Jr., Silva, J. L., and Foguel, D. (1999) *J. Mol. Biol. 287*, 527−538.
58. Kocsis, E., Greenstone, H. L., Locke, E. G., Kessel, M., and Steven, A. C. (1997) *J. Struct. Biol. 118*, 73−82.
59. Lanman, J., Tuma, R., and Prevelige, P. E. (1999) *Biochemistry 38*, 14614−14623.
60. Prevelige, P. E., Jr., Thomas, D. H., Aubrey, K. L., Towse, S. A., and Thomas, G. J., Jr. (1993) *Biochemistry 32*, 337−343.
61. Tuma, R., Prevelige, P. E., Jr., and Thomas, G. J., Jr. (1996) *Biochemistry 35*, 4619−4627.
62. Kelly, L., and Holladay, L. A. (1990) *Biochemistry 29*, 5062−5069.
63. Christensen, T., Svensson, B., and Sigurskjold, B. W. (1999) *Biochemistry 38*, 6300−6310.
64. Vetter, I. R., Baase, W. A., Heinz, D. W., Xiong, J.-P., Snow, S., and Matthews, B. W. (1996) *Protein Sci. 12*, 2399−2415.
65. Lashuel, H. A., Lai, Z., and Kelly, J. W. (1998) *Biochemistry 37*, 17851−17864.
66. Lee, Y. K., Brewer, J. W., Hellman, R., and Hendershot, L. M. (1999) *Mol. Biol. Cell 10*, 2209−2219.
67. Schulteis, C. T., Nagaya, N., and Papazian, D. M. (1998) *J. Biol. Chem. 273*, 26210−26217.
68. Serwer, P., and Pichler, M. E. (1978) *J. Virol. 28*, 917−928.
69. Aramli, L. A., and Teschke, C. M. (1999) *J. Biol. Chem. 274*, 22217−22224.
70. Sugihara, J., and Baldwin, T. O. (1988) *Biochemistry 27*, 2872−2880.
71. Choi, H., Tang, C.-K., and Tu, S.-C. (1995) *J. Biol. Chem. 270*, 16813−16819.
72. Smith, D. H., Berget, P. B., and King, J. (1980) *Genetics 96*, 331−352.
73. Betts, S., Haase-Pettingell, C., and King, J. (1997) *Adv. Protein Chem. 50*, 243−264.
74. Chen, B., and King, J. (1991) *Biochemistry 30*, 6260−6269.
75. Danner, M., Fuchs, A., Miller, S., and Seckler, R. (1993) *Eur. J. Biochem. 215*, 653−661.
76. Miller, S., Schuler, B., and Seckler, R. (1998) *Protein Sci. 7*, 2223−2232.
77. Wetzel, R. (1992) in *Stability of Protein Pharmaceuticals, Part B: In Vivo Pathways of Degradation and Strategies of Protein Stabilization* (Ahern, T. J., and Manning, M. C., Eds.) pp 43−88, Plenum Press, New York.
78. Chrunyk, B. A., Evans, J., Lillquist, J., Young, P., and Wetzel, R. (1993) *J. Biol. Chem. 268*, 18053−18061.
79. Wetzel, R., and Chrunyk, B. A. (1994) *FEBS Lett. 350*, 245−248.
80. Wetzel, R., Perry, L. J., and Veilleux, C. (1991) *Biotechnology (N.Y.) 9*, 731−737.
81. Alber, T., Sun, D.-p., Nye, J. A., Muchmore, D. C., and Matthews, B. W. (1987) *Biochemistry 26*, 3754−3758.
82. Gray, T. M., and Matthews, B. W. (1987) *J. Biol. Chem. 262*, 16858−16864.
83. Grutter, M. G., Gray, T. M., Weaver, L. H., Wilson, T. A., and Matthews, B. W. (1987) *J. Mol. Biol. 197*, 315−329.

BI991956T