Aggregation and Assembly of Phage P22 Temperature-Sensitive Coat Protein Mutants in Vitro Mimic the in Vivo Phenotype†

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ABSTRACT: Aggregation is a common side reaction in the folding of proteins which is likely due to inappropriate interactions of folding intermediates. In the in vivo folding of phage P22 coat protein, amino acid substitutions that cause a temperature-sensitive-folding (*tsf*) phenotype lead to the localization of the mutant coat proteins to inclusion bodies. Investigated here is the aggregation of wild-type (WT) coat protein and 3 *tsf* mutants of coat protein. The *tsf* coat proteins aggregated when refolded in vitro at high temperature. If the *tsf* coat proteins were refolded at 4 °C, they were able attain an assembly active state. WT coat protein, on the other hand, did not aggregate significantly even when folded at high temperature. The refolded *tsf* mutants exhibited altered secondary and tertiary structures and had an increased surface hydrophobicity, which may explain the increased propensity of their folding intermediates to aggregate.

The amino acid sequence of a protein dictates its folding and assembly pathways, as well as its final structure (*1*). An unfortunate consequence of the primary sequence determination of folding and structure is that single amino acid substitutions can lead to profound changes in the folding of a protein and its final structure. For instance, in sickle cell anemia, HbS polymerizes because of the glu6 to val substitution (*2*, *3*). In osteogenesis imperfecta, many mutations lead to a single amino acid change of a glycine to a bulkier amino acid in type I collagen, resulting in weakened collagen fibrils (*4*, *5*). Another consequence of amino acid substitutions in the primary sequence is alterations in the conformations of folding intermediates, which may cause amyloid fiber or inclusion body formation, which are forms of aggregated protein. Diseases caused by abnormal folding or unfolding, followed by aggregation, are known as conformational diseases and are caused by such unrelated proteins as tranthyretin, immunoglobulin light chains, and lysozyme (*6*−*9*). Single amino acid substitutions in these proteins which lead to the disease state destabilize the protein to heat or acid, causing its folding intermediates to aggregate.

During the folding process, folding intermediates may interact inappropriately before reaching the native state. These off-pathway aggregation reactions occur frequently when a protein is expressed in a heterologous host. Aggregation reactions have serious ramifications for the biotechnology industry since it can be difficult to express sufficient quantities of a protein in the native, active state to allow for efficient drug production (*10*−*12*). Single amino acid substitutions can shift the folding from the productive pathway to off-pathway aggregation. This result has been observed for the bacteriophage P22 tailspike protein where mutations that lead to a temperature-sensitive phenotype cause the specific off-pathway aggregation of tailspike folding intermediates (*13*−*18*). The folding intermediates of several other proteins, such as interluekin-1β (*19*) and bovine growth hormone (*20*) show similar tendencies to aggregate.

Proteins that are aggregation prone often use folding assistants, known as molecular chaperones, to facilitate proper folding (*21*). In bacteria, the major cytosolic chaperone system is the GroEL and GroES complex and is involved in the folding of about 10−15% of bacterial proteins (*22*, *23*). Molecular chaperones, such as GroEL/ES, bind to nonnative folding intermediates and prevent the improper association of the intermediates, thereby resulting in a higher yield of native protein.

The protein investigated here is the coat protein of bacteriophage P22. The $T = 7$ icosahedral shell of bacteriophage P22, a dsDNA bacteriophage of *Salmonella*, consists of 420 coat protein subunits, each of which is a 47 kDa polypeptide of 430 amino acids (*24*, *25*). During assembly, coat protein interacts with 150−300 molecules of a 33 kDa scaffolding protein in a polymerization reaction which produces the procapsid, a precursor capsid (*26*−*30*). Assembly of the procapsid proceeds in a nucleation-limited reaction by the addition of monomers of coat protein to the growing edge of the partially formed capsid (*31*). Also during assembly of the procapsid, three pilot proteins and a portal protein complex are incorporated (*24*, *32*). The scaffolding protein exits through holes present in the procapsid lattice while the packaging of DNA occurs through the portal vertex (*26*, *27*, *33*). The packaging of DNA is accompanied by simultaneous expansion of the capsid lattice into the mature capsid which is characterized by a 15% increase in diameter, a change in shape from spherical to icosahedral, and the closure of the holes in the lattice (*27*, *34*, *35*).

Eighteen amino acid substitutions at 17 sites throughout the sequence of phage P22 coat protein have been identified that cause production of phage to be temperature-sensitive

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(*36*, *37*). These temperature-sensitive-folding (*tsf*)[1] mutants of coat protein sharply reduce the yield of soluble protein in vivo at the nonpermissive temperature, and the newly synthesized coat polypeptides aggregate to form inclusion bodies prior to reaching the assembly competent state. Remarkably, in vivo all 18 *tsf* mutants are rescued by overproduction of the chaperones GroEL and GroES (*38*). High intracellular concentrations of GroEL and GroES inhibit aggregation by competing for the off-pathway folding intermediates. Interaction with GroEL/S allows the *tsf* coat proteins to fold into an assembly competent conformation (*39*).

When the folding of 5 of the *tsf* mutants was investigated in vitro, we found that they folded into dimers and trimers in contrast to WT coat protein which folded into monomers (*40*−*42*). The WT coat protein monomers were assembly competent while the refolded *tsf* mutants were not (*41*). The WT and *tsf* coat polypeptides were found to aggregate with the same propensity and temperature-dependence. Although differences were found when comparing WT and *tsf* coat proteins, the in vitro folding and assembly reactions did not mimic the in vivo phenotype. Here in vitro experiments are described where the results simulate the in vivo phenotype; that is, when that *tsf* coat proteins were folded at low temperature, they were assembly competent, but when refolded at high temperature, the *tsf* mutants aggregated.

MATERIAL AND METHODS

Chemicals. Ultrapure guanidine hydrochloride (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 purchased from common sources.

Buffer. The buffer used in all of the experiments was 20 mM sodium phosphate, pH 7.6. For procapsid preparations and storage of shell stocks, the buffer used was 50 mM Tris base, 25 mM NaCl, and 2 mM EDTA, adjusted to pH 7.6 with HCl.

Purification of tsf Coat Proteins. The *tsf* coat proteins used in the refolding experiments were obtained from empty procapsid shell stocks that were prepared as previously described (*29*, *40*, *42*). Briefly, *Salmonella typhimurium* were infected at 28 °C with bacteriophage P22 carrying a temperature-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 of buffer. The cells were lysed by a freeze/thaw cycle, treated with RNase and DNase, and spun at 45 000 rpm in a Ti60 rotor in a Beckman L7-65 for 35 min to pellet the procapsids. After suspension of the procapsids by shaking at 4 °C overnight, the procapsids were passed over a Sephacryl S1000 (Pharmacia-LKB) 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. The protein concentration was determined by absorbance 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 (*43*). The purified empty procapsid shells were suspended in buffer and stored indefinitely at 8 mg/mL at 4 °C.

Refolding of Coat Protein by Dialysis. Empty procapsid shells were unfolded by incubation in 6.75 M urea. 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 denatured samples were loaded into the microdialyzer (Gibco-BRL Life Technologies) and dialyzed at 4 °C at rate of approximately 45 mL/h for a period of about 3 h or until residual urea was undetectable by refractive index. The protein concentration was determined by absorbance at 280 nm.

Refolding of Coat Protein by Rapid Dilution. Coat protein was unfolded in urea as described above. To initiate refolding, the urea was rapidly diluted 20-fold from 6.75 to 0.34 M urea and a final coat protein concentration of 0.1 mg/mL.

Procapsid Assembly Reactions. To assemble coat protein into procapsids, 100 μL of coat protein refolded by dialysis at 0.7 mg/mL was mixed with 10 μL of scaffolding protein at 10 mg/mL at 20 °C in the SLM Aminco-Bowman 2 spectrofluorometer. The reaction was monitored by the increase in 90° light scattering at 500 nm with the band-passes set to 4 nm. The reactions were diluted with agarose gel sample buffer (40 mM Tris base, 1 mM EDTA, 20% sucrose, pH 8.3 with acetic acid) and loaded onto 1.2% Seakem HGT agarose gel made with the same buffer without sucrose and run at 50 V for 4−5 h at room temperature. The gel was stained with Coomassie blue.

Native Gel Electrophoresis. Coat protein samples were refolded by dialysis or rapid dilution as described above. Samples for the native gel were prepared by combining a portion of refolded coat protein with 3× 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 (*44*). The gel was run at 10 mA constant current and 4 °C. The protein bands were visualized by Coomassie stain.

Tryptophan Fluorescence Spectra. Coat protein samples were dialyzed at 4 °C as described. A fluorescence emission spectrum from 310 to 400 nm with excitation set at 280 nm and band-passes at 4 nm was obtained using a SLM Aminco-Bowman 2 spectrofluorometer maintained at 20 °C. The spectra were normalized so that the emission maxima equaled 1.

BisANS Fluorescence Spectra. A stock solution of bisANS was prepared in buffer and the bisANS concentration determined using the extinction coefficient of 16 790 L mol^{-1} cm^{-1} at 385 nm (*45*). Empty procapsid shells were denatured and folded by dialysis as above. Coat protein samples at 0.7 μM were incubated with bisANS at a concentration of 12.5 μM. An emission scan from 425 to 625 nm was performed with the fluorescence spectrophotometer set at an excitation wavelength of 390 nm and band-passes of 4 nm. A constant temperature of 20 °C was maintained by a thermostated cuvette holder.

BisANS Binding Assay. BisANS binding to WT and *tsf* coat proteins was determined using a double titration method

[1] Abbreviations: WT, wild-type; *tsf*, temperature-sensitive-folding; bisANS, 1,1′-bis(4-anilino)naphthalene-5,5′-disulfonic acid; GuHCl, guanidine hydrochloride.

with the excitation wavelength set at 390 nm and the emission wavelength at 495 nm (*46*, *47*). In one titration the bisANS concentration is fixed and the concentration of coat protein is varied. The *y*-intercept of a plot of $1/F$ vs 1/[coat] is $1/F_{max}$, where F_{max} is the maximum fluorescence intensity. F_{max}/[bisANS] is the maximum fluorescence units/μM bisANS bound to coat protein. In the second titration, the coat protein concentration was held at about 1 μM and the bisANS concentration varied from 2 to 75 μM. The fluorescence of these samples was corrected for the inner filter effect, which becomes substantial at high [bisANS], as described in Lakowicz (*56*). The fluorescence values were converted into μM bisANS bound/μM coat protein. A plot of μM bisANS bound/μM coat protein vs free [bisANS] was analyzed with Kaliedagraph (Abelbeck Software) using the formula $y = n[\text{bisANS}]/(K_d + [\text{bisANS}])$, where n is the number of sites and K_d is the dissociation constant (*46*). The bisANS binding isotherm of WT coat protein showed positive cooperativity and therefore was analyzed with the Hill equation, $\log F/F_{max} = n \log[\text{bisANS}] - \log K_d$ (*54*).

Proteolysis of Refolded Coat Protein. Lyophilized chymotrypsin attached to agarose beads was rehydrated and washed three times with cold buffer, and a final 1:1 slurry of beads to buffer was kept on ice prior to use. Refolded coat protein was prepared by dialysis at 4 °C as described above, and the concentration differences were corrected before further analysis. For each digest, a 50 μL sample of refolded coat protein at 0.33 mg/mL was combined with 5 μL of the 1:1 slurry of chymotrysin−agarose in a microcentrifuge tube and placed on a Nutator at room temperature (~22 °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 3× SDS sample buffer, run on 15% SDS gels, and silver stained (*48*). Digestion periods are described in the figure. The band of full-length coat protein was quantified using a Kodak EDAS 120 system.

Circular Dichroism of Refolded Coat Protein. Samples were prepared in the microdialyzer at 4 °C as described. The protein concentrations were determined using the extinction coefficient for coat protein of 0.957 mL mg^{-1} cm^{-1} at 280 nm (*41*). The spectra, the average of 15 scans, were taken with a Jasco J-715 circular dichroism spectrophotometer thermostatted at 20 °C with a Peltier accessory from 250 to 190 nm with the following settings: scan rate of 100 nm/min, response time of 2 s, a bandwidth of 0.2 nm, and a step resolution of 0.5 nm. A 2 mm path length cell was used.

RESULTS

In vivo, when cells are infected at permissive temperatures with phage that carry a *tsf* amino acid substitution in coat protein, folding and assembly proceed normally and a burst of ~100 phage/cell is produced (*36*, *39*). When the cells are instead infected at nonpermissive high temperatures, the *tsf* coat proteins aggregate and are sequestered in inclusion bodies (*36*, *39*). GroEL and GroES rescue the folding of the *tsf* coat proteins by binding to a thermolabile and aggregation-prone folding intermediate and thereby prevent aggregation from occurring. To understand the effect of the *tsf* amino acid substitutions on the folding, aggregation, and assembly reactions of coat protein, conditions under which each reaction would predominate in a controllable fashion needed to be determined. Described in this report are conditions where in vitro aggregation and assembly of WT and *tsf* coat proteins mimic the phenotype observed in vivo.

In previous experiments where the in vitro folding and aggregation of the *tsf* coat proteins have been studied, several buffers were used. In the work of Teschke and King (*41*), a buffer composed of 25 mM Tris base, pH 7.6, 50 mM NaCl, and 2 mM EDTA was used because this buffer has been shown to permit the folding of WT coat protein into an assembly competent conformation (*29*, *40*, *50*). In this buffer, the refolded *tsf* coat proteins showed significant conformational differences compared to WT coat protein, but even when refolded at low temperature in vitro, the *tsf* coat proteins were assembly incompetent. Additionally, WT coat protein and the *tsf* coat proteins aggregated identically in a temperature-dependent manner. Thus, this Tris buffer did not meet the desired criterion of mimicking the in vivo phenotype.

Galisteo, Gordon, and King (*42*) used 20 mM potassium phosphate, pH 7.6, and 25 mM NaCl and again observed changes in conformation between *tsf* and WT coat proteins. However, neither the potential for aggregation nor the ability of the *tsf* coat protein to assemble into procapsids was investigated. This buffer has a higher ionic strength than the Tris buffer used in Teschke and King (*41*), which is a concern since procapsid assembly is sensitive to ionic strength (*50*). Speed, Wang, and King (*16*) found that 0.4 M urea in 40 mM sodium phosphate, pH 7.6 at 30 °C, caused WT coat protein to be aggregation prone. However, in vivo at 30 °C only 5% of WT coat protein is found in inclusion bodies and only about 25% of WT coat protein aggregates at 39 °C (*39*). In 0.4 M urea WT coat protein is just beginning to unfold, as determined by equilibrium refolding/unfolding experiments (data not shown), and so partial unfolding is likely to be the reason that WT coat protein was particularly aggregation prone in the experiments by Speed et al. (*16*). Here I report the results of in vitro assembly and aggregation studies of WT and *tsf* coat proteins when refolded from urea in 20 mM sodium phosphate, pH 7.6. This buffer was chosen because it has the same ionic strength as the Tris buffer mentioned above and because Speed, Wang, and King (*16*) were able to cause aggregation of coat protein in a phosphate buffer.

Assembly of WT and tsf Coat Proteins. A measure of proper folding of coat protein is the ability to assemble into a procapsid (*29*, *40*, *41*, *51*, *52*). To determine the assembly competence of WT and *tsf* coat proteins after being refolded by dialysis at permissive temperature (4 °C) in 20 mM phosphate buffer, each species of refolded coat protein was mixed with scaffolding protein at 20 °C. The reaction was monitored by the increase in 90° light scattering at 500 nm (Figure 1). WT coat protein assembled as expected (*49*, *51*, *52*), and the *tsf* coat protein mutants were also competent for assembly, albeit with slower kinetics than WT coat protein.

Since light scattering detects any increase in the number of large particles in solution, it was possible that this buffer caused the coat proteins to aggregate rather than to assemble into procapsids when incubated at 20 °C. To confirm that procapsids had formed during the reaction, the assembly reactions were run on an agarose gel after incubation overnight at 20 °C. To identify the position of each procapsid

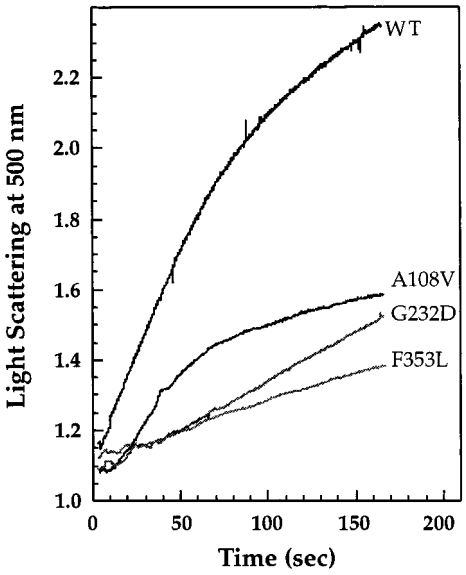

FIGURE 1: Assembly of WT and *tsf* coat proteins. Coat proteins that had been refolded by dialysis, as described in the Materials and Methods, were mixed with scaffolding protein, and the assembly of procapsids was monitored by the increase in light scattering at 500 nm at 20 °C. The species of coat protein is indicated on the right side of the figure. The coat protein concentration was 15 μM, and the scaffolding protein concentration was 30 μM.

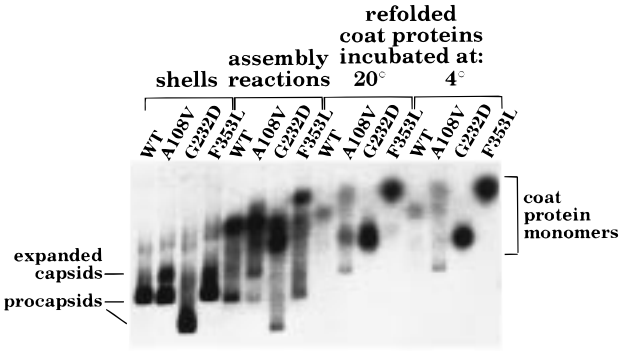

FIGURE 2: Assembly reactions yield procapsids. A sample of each assembly reaction shown in Figure 1 was run on a 1.2% agarose gel in the lanes labeled assembly reactions. Empty procapsids shells run as markers for procapsids are shown in the lanes on the left side of the gel. The proteins incubated at either 4 or 20 °C without the addition of scaffolding protein are shown on the right side of the gel. Coat protein monomers, indicated on the right side of the gel, migrate on agarose gels more slowly than procapsids.

species on the agarose gel, samples of the isolated empty procapsid shell stocks were used as procapsid markers (Figure 2). The lanes of the assembly reactions of the refolded coat proteins each contain a band that ran in the same position as the corresponding species of procapsids. Thus, the *tsf* coat proteins were able to assemble into procapsids. The procapsids of the mutant G232D ran with higher mobility than WT procapsids on the gel, consistent with the change of gly to asp. The stock solution of empty procapsid shells of the mutant A108V contained some expanded capsids which occurred as a result of the extraction of scaffolding protein during purification. These expanded capsids are not dissociated by incubation with 6.75 M urea which was used to unfold coat protein and are seen on the gel as a band of lower mobility than procapsids. The refolded coat proteins without the addition of scaffolding protein were

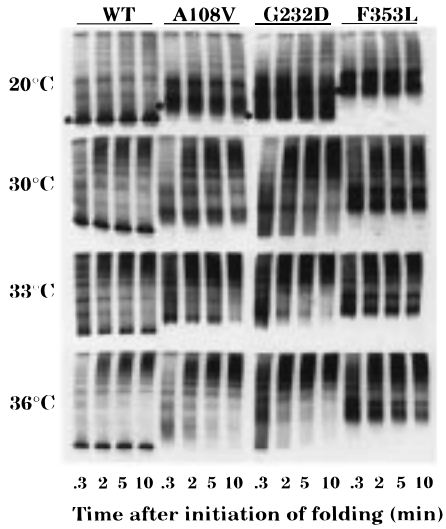

FIGURE 3: Aggregation during folding. WT or *tsf* coat proteins that were denatured in urea were diluted into buffer at the temperatures indicated on the left of the figure. Samples were taken from the refolding reactions at the times indicated on the bottom of the figure and chilled on ice, and the reactions were run on a native gel. The stars in the 20 °C row indicate the position of the folded form of WT and *tsf* coat proteins. Aggregates are seen as the ladder of bands of slower mobility on the native gel as compared to the folded coat proteins.

also incubated at either 20 or 4 °C to determine the extent of loss of protein due to aggregation with the prolonged exposure to 20 °C, but little protein loss was seen when compared to the bands in the 4 °C lanes. This control also showed that scaffolding was required for procapsid assembly. Thus, the results of this agarose gel indicate that both the refolded WT and *tsf* coat proteins were folded into an assembly competent conformation in the 20 mM sodium phosphate buffer and that the increase in light scattering (Figure 1) was not due to aggregation.

Aggregation of the tsf Coat Proteins. Since the *tsf* coat proteins were assembly competent in 20 mM phosphate when refolded at a permissive temperature, next investigated was whether WT and *tsf* coat proteins aggregated when refolded by rapid dilution at a variety of temperatures. At times after the initiation of folding at a particular temperature, an aliquot of the refolding reaction was diluted with native gel sample buffer in a tube held on ice. Aggregation was observed by running the samples on a native gel. On native gels, aggregates are seen as a ladder of bands of decreasing mobility compared to the band of folded protein, which is indicated in Figure 3 by a star (★) (*14*, *15*). In Figure 3, when folding of WT and *tsf* coat proteins was initiated at 20 °C, some aggregates were seen in each of the folding reactions. Since the disappearance of bands from the ladder of aggregating coat proteins was gradual, the rate of aggregation at 20 °C was slow. The intensity of the band corresponding to each of the folded proteins appeared constant with time, suggesting that at 20 °C the aggregation was likely due to incorrectly folded polypeptides which did not run in the monomer position on the native gel. When folding was initiated at 30 °C, the intensity of the band of folded WT coat protein remained constant and the aggregated forms seemed to originate from species of decreased mobility, as these were the bands that disappeared with time. This result suggests that once WT coat protein folded into the

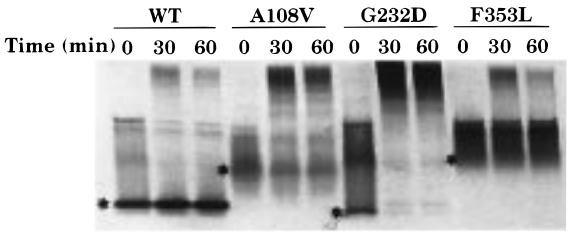

FIGURE 4: Temperature shift after folding on ice. WT and *tsf* coat proteins were refolded from urea by rapid dilution with buffer on ice. At 30 or 60 min after folding was initiated, a sample was shifted to 33 °C for 10 min. At 10 min the sample was diluted with ice cold sample buffer and run on a native gel. The zero time point was held on ice and never shifted to 33 °C. The stars indicate the position of the folded form of each of the coat proteins.

monomeric conformation, it was stable. In contrast, at 30 °C the *tsf* coat protein mutant G232D aggregated extensively and the aggregates appeared to originate from the folded monomeric form of the protein since the band corresponding to monomer decreased in intensity with time. At 30 °C, A108V appeared to aggregate from the monomeric species to a small extent, but the monomeric form of F353L seemed stable. At 33 °C, extensive aggregation of A108V occurred during the folding reaction, again beginning with the band corresponding to the folded form of the protein. Monomeric WT and F353L coat proteins were resistant to incubation at 33 °C since the intensity of the monomeric band was constant with time. G232D aggregated rapidly at 33 °C. Finally, when refolding was initiated at 36 °C, F353L began to aggregate. The band of folded monomeric WT coat protein remained constant in intensity even when refolding was performed at 36 °C, and the aggregation that occurred in this reaction again came from bands of decreased mobility rather than the folded protein. The more aggregation prone mutant proteins, A108V and G232D, aggregated rapidly and extensively at 36 °C. For comparison of a permissive folding reaction initiated and held on ice, see Figure 4.

Betts and King (*53*) reported that an incubation of only 1 min on ice during folding of phage P22 tailspike protein prevented aggregation of a thermolabile folding intermediate when the folding reaction was shifted to a higher temperature. To determine if such a thermolabile intermediate in the folding of coat protein could be rescued from aggregation, folding was initiated on ice and, after incubation for 30 or 60 min, the samples were shifted to 33 °C. After 10 min at 33 °C, an aliquot of WT or the *tsf* coat proteins was diluted with native gel sample buffer on ice and run on a native gel (Figure 4). Again, the stars (★) in Figure 4 mark the position of the folded proteins. The zero time point refers to protein refolded on ice and never shifted to 33 °C. The intensity of the band corresponding to folded WT coat protein was constant, and the aggregation that did occur appeared to come from bands with decreased mobility compared to the band of folded WT coat protein. There was more aggregate in the WT sample folded on ice for 30 min than in the one folded for 60 min, suggesting that there may have been some residual slowly folding intermediates that were aggregation prone. A similar result was seen with the *tsf* mutant F353L, although F353L appeared to be somewhat more disposed to aggregation than WT coat protein. The mutants A108V and G232D almost completely aggregated upon the temperature shift to 33 °C and showed a decrease in intensity of the band

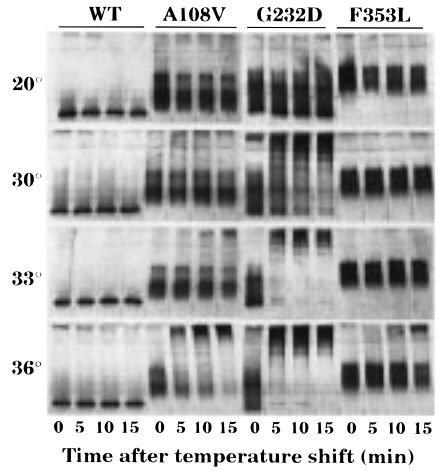

FIGURE 5: Temperature shift after folding by dialysis. WT and *tsf* coat proteins were refolded by dialysis at 4 °C until the urea was removed from the sample. A aliquot of each was shifted to the temperatures shown on the left of the figure, and at the time indicated on the bottom, a sample was removed to tubes held on ice. The samples were run on a native gel. Aggregates are the ladder of bands of slower mobility on the gel as compared to the folded coat proteins.

that corresponds to the folded protein indicating that the folded state of these *tsf* coat proteins was aggregation prone.

One possibility for the tendency of *tsf* mutant proteins to aggregate was the 0.334 M residual urea in the refolding reaction. To avoid residual urea, WT and *tsf* coat proteins were refolded by dialysis and temperature shift experiments were done. Dialysis for 3 h was determined in the experiment shown in Figure 1 to be sufficient to produce folded assembly competent coat protein monomers. Presented in Figure 5 are the results of shifting the refolded coat proteins from 4 °C to temperatures from 20 to 36 °C. An incubation of 15 min at 20 °C did not cause any appreciable aggregation of any of the coat proteins. However, at 30 °C G232D began to aggregate within 5 min, at 33 °C A108V aggregated, and at 36 °C F353L became aggregation prone. WT coat protein was resistant to aggregation at all temperatures. Thus, the *tsf* coat proteins are aggregation prone at high temperatures, even when folded at permissive temperatures.

Tertiary Structure of the tsf Coat Protein Is Altered. The mobilities of WT coat protein and *tsf* mutants that were refolded at 4 °C on the native gel shown in Figure 5 (0 min time points) are different. Previously, these changes in mobility were explained as a shift of the *tsf* mutants to dimeric and trimeric folded states from the monomeric state into which WT coat protein folds (*41*, *42*). Recently, we have determined by analytical ultracentrifugation that *tsf* mutants are primarily monomeric (C.M.T. and E. Braswell, unpublished results). Thus, it was probable that alterations in tertiary and secondary structure were responsible both for their differences in mobility on a native gel and for the changes in assembly kinetics and aggregation tendencies of the *tsf* coat proteins.

To determine if there are changes in their tertiary structure, the tryptophan emission spectra of the WT and *tsf* coat proteins at 20 °C were determined after refolding by dialysis at 4 °C (Figure 6). For all of the following conformational analyses, the temperature of 20 °C was chosen because the aggregation reaction would not interfere at this temperature;

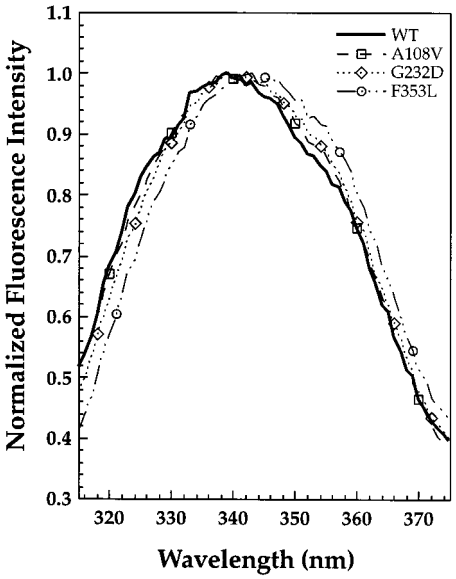

FIGURE 6: Fluorescence spectra of refolded coat proteins. WT and *tsf* coat proteins were refolded by dialysis at 4 °C. The spectra were taken at 20 °C with an excitation wavelength of 280 nm as described in the Materials and Methods.

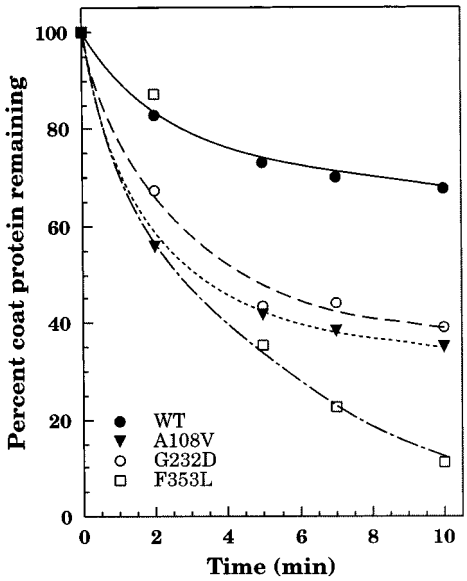

FIGURE 7: Protease sensitivity of refolded coat proteins. WT and *tsf* coat proteins were refolded by dialysis at 4 °C. The coat proteins were incubated at room temperature with chymotrypsin attached to beads for the times indicated. The rate at which full-length coat protein was digested by chymotrypsin was determined by quantification of the intensity of the band of full-length coat protein on an SDS−polyacrylamide gel. The lines are added to aid the eye.

Table 1: Binding of BisANS to WT and *tsf* Coat Proteins

	K_d (μM)	no. of sites	Hill coeff
WT	76.6 ± 22.8		1.4 ± 0.2
A108V	15.3 ± 4.8	7.2 ± 4.8	
G232D	16.2 ± 7.6	6.4 ± 4.0	
F353L	14.9 ± 3.0	16.8 ± 12.8	

nevertheless, the proteins were poised to aggregate if the temperature was shifted up. A shift in the emission maximum to longer wavelengths, as compared to the refolded WT coat protein spectrum, was seen for G232D and F353L, which is consistent with increased solvent exposure of the tryptophans. The spectrum of the *tsf* mutant A108V was similar to that of WT coat protein. These results were consistent with previously described experiments using the different buffers described above (*41*, *42*).

Since it was likely that the tryptophans of some of the *tsf* coat proteins were more solvent exposed than those of WT coat protein, it was possible that the proteins would exhibit increased surface hydrophobicity. The binding of the hydrophobic dye bisANS was measured to analyze the hydrophobicity of WT and *tsf* coat proteins (Table 1). When WT coat protein was incubated with bisANS, the binding isotherm showed positive cooperativity (data not shown), so the binding was analyzed with the Hill equation (*54*). The binding of bisANS to the *tsf* coat proteins did not show cooperativity and was analyzed using Scatchard analysis. The binding of bisANS to the *tsf* coat proteins showed higher affinity, and more bisANS molecules bound to each *tsf* coat protein than WT coat protein. These data indicate that the tertiary structure of the *tsf* coat proteins was altered so that the tryptophans were more solvent exposed and had more hydrophobic surface than WT coat protein.

As an additional probe of changes in tertiary structure, the rate of digestion of full length coat protein with protease was determined. In Figure 7, the progress of digestion of WT and *tsf* coat proteins with chymotrypsin is shown. WT coat protein was most resistant to protease with 67% of the full length protein remaining after 10 min. The *tsf* mutant proteins were dramatically more sensitive to protease. At 10 min of incubation with chymotrypsin, F353L had only 11% remaining full length protein. A108V and G232D had 35−40% of the protein left after proteolysis. Additionally, different peptides were seen when the protease digests of *tsf* coat proteins were run on SDS gels compared to the digest of WT coat protein (data not shown). These data indicate that the tertiary structure of the *tsf* coat proteins has been changed by the amino acid substitutions and were likely less compact than WT coat protein.

Secondary Structure of the tsf Coat Proteins Is Altered. Since there were changes in the tertiary structure of the *tsf* coat protein mutants, it was possible that there could be changes in the secondary structure as well. To investigate the secondary structure of the WT and *tsf* coat proteins, the proteins were refolded by dialysis at 4 °C and a circular dichroism spectrum of each taken at 20 °C. In Figure 8, the circular dichroism spectra of WT and the *tsf* coat proteins are shown. The spectrum of WT coat protein was similar to previously published spectra (*52*). The spectra of the mutant coat proteins were altered from the spectrum of WT coat protein and appear to be different than the spectra previously taken of *tsf* coat mutants in the other buffers described above (*41*, *42*). These changes in secondary structure suggest that the alterations in tertiary structure described above are not simply due to rearrangement of secondary structural elements.

DISCUSSION

Proper folding, assembly, and aggregation have been shown here to depend on the temperature and conditions of the refolding reaction. At low temperature, folding into the assembly competent state occurred. When scaffolding protein was added to the refolded coat proteins, procapsids were

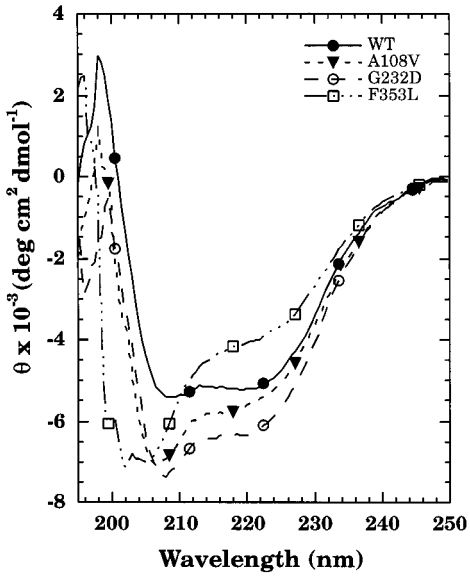

FIGURE 8: Circular dichroism spectra of refolded coat proteins. WT and *tsf* coat proteins were refolded by dialysis at 4 °C. The circular dichroism spectra were taken at 20 °C using a Jasco J-715 circular dichroism spectrometer as described in the Materials and Methods.

formed. If the refolding reaction was done at high temperatures, aggregation became the predominant reaction. When *tsf* coat proteins refolded at permissive temperature were shifted to high temperature, aggregation was seen. Thus, refolded *tsf* coat proteins must thermolabile themselves and in equilibrium with an aggregation prone intermediate.

Effect of the Refolding Condition on the Conformation of WT and tsf Coat Proteins. Substantive differences were observed when the conformations of WT and *tsf* coat proteins that were folded in 20 mM phosphate buffer were compared to the conformations seen in buffers used in previous reports (*41*, *42*). For example, the circular dichroism spectra of the WT and *tsf* coat proteins when folded in the Tris buffer of Teschke and King (*41*) had very different shapes than shown here. However, the folded *tsf* coat proteins were not assembly competent and the aggregation propensities of WT and *tsf* coat proteins were the same in that buffer. The circular dichroism spectrum of each of the *tsf* coat proteins in the phosphate/NaCl buffer used by Galisteo et al. (*42*) had a shape similar to that of F353L shown here, with the peak of greatest negative ellipticity centered around 203 nm. The emission maximum of the fluorescence spectrum of WT coat protein was shifted to lower wavelengths in the buffer used here as compared to the buffer used by Galisteo et al. (*42*), indicating that the tryptophans were in a less polar environment. Additionally, WT coat protein when folded in the Tris buffer (*41*) bound bisANS with higher affinity than was seen here, which suggests an increase in the hydrophobicity of the protein in the Tris buffer. Thus, the conformation of coat protein is dependent on the buffer used when refolding. Perhaps it is not surprising that coat protein is flexible enough to acquire these different conformations since it must adopt several conformations during assembly to form a closed, spherical capsid (*30*).

Assembly Is Affected by the tsf Amino Acid Substitutions. The *tsf* coat proteins were able to fold in the phosphate buffer at 4 °C into a state that was assembly competent and capable of forming procapsids of normal size. The kinetics of the in vitro assembly reaction of the *tsf* mutants was slower than that of WT coat protein, consistent with our results from in vivo studies where the *tsf* mutants assemble more slowly than WT coat protein, even at permissive temperatures (*39*). Given that the changes in secondary and tertiary structure of the *tsf* coat proteins refolded in vitro were substantial when compared to refolded WT coat protein, there are two likely possibilities for the assembly competence of the *tsf* coat proteins. There could be a population of WT-like, assembly competent molecules in solution in equilibrium with noncompetent *tsf* coat proteins, and during assembly the equilibrium is shifted so that the assembly incompetent coat proteins undergo a conformational change to become assembly competent. Alternatively, the assembly reaction of coat protein could be adaptive enough to accept the altered molecules. We have investigated the effect of the amino acid substitutions on the structure of procapsids formed in vivo at permissive temperature and have found that every *tsf* amino acid substitution causes changes in the ability of the capsids to expand, as well as some coat protein secondary structure changes within the capsid lattice (C. M. Capen and C.M.T., unpublished data). These results suggest that it is more probable that the *tsf* coat proteins having altered conformations are competent for assembly.

Aggregation of the tsf Coat Proteins. Aggregation of the *tsf* coat proteins occurred both during folding at high temperature and when the temperature was shifted after the protein was first folded at 4 °C. The shift to high temperature most likely caused the *tsf* proteins to unfold to the aggregation prone intermediate that exists in the folding pathway. Once folded, WT coat protein appeared to be resistant to aggregation. Thus, the barrier between the WT folded monomeric state and the aggregation prone intermediate must be substantially higher than that of the *tsf* coat proteins. This is similar to what has been hypothesized to occur in amyloidogenic diseases where a conformational change due to an environmental change or an amino acid substitution destabilizes the folded state to an aggregation prone intermediate (*53*, *55*).

Model for the Folding of Coat Protein. On the basis of the data presented here, we propose the following working model of the folding and assembly of coat protein. (Figure 9). In this model, WT coat protein folds from the unfolded state through intermediates I1 and I2 to the stable folded subunit. When folding at higher temperatures, a portion of the folding polypeptide partitions off-pathway to the aggregation prone $I^{off-pathway}$ intermediate. Even at high temperature, WT coat polypeptides are able to fold properly if they partition down the correct pathway. Once folded into the soluble subunit, a shift to high temperature does not cause WT coat protein to aggregate. On the basis of the native gel analysis of the aggregation reaction, WT coat protein is likely to aggregate via a dimer of the $I^{off-pathway}$ intermediate. Further experiments will be needed to determine the multimeric composition of the aggregating species.

The folding of the *tsf* coat proteins at permissive temperature results in the formation of an assembly competent subunit that has an altered conformation. From experiments not presented here, we have determined that the kinetics of formation of the intermediate I2 of the *tsf* coat protein has been slowed by the *tsf* amino acid substitutions (C.M.T., unpublished results). The rate of formation of the I1

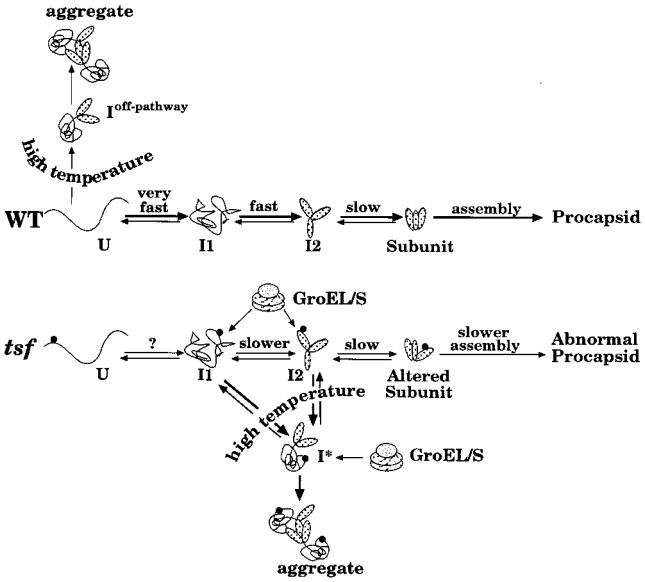

FIGURE 9: Model for the folding of WT and *tsf* coat proteins. The upper part of the diagram shows the model for the folding of WT coat protein, and the lower half, the folding of the *tsf* coat proteins. The thickness of the arrows indicates the direction of the equilibrium. The dot denotes a *tsf* amino acid substitution. WT coat protein folds efficiently into a subunit which can assemble into a normal procapsid. The *tsf* coat proteins fold into an altered conformation that is assembly competent but leads to an altered, yet functional, procapsid. High temperature causes a portion of WT coat polypeptides to fold into an off-pathway intermediate that is aggregation prone. In the *tsf* coat proteins, the concentration of the I* intermediate increases at high temperature causing an accumulation of aggregates. The folded *tsf* subunit is easily shifted back to the intermediate forms when the temperature is increased, which results in increased aggregation. In vivo, GroEL/S intercede in the folding of the *tsf* mutants through interaction with one of the folding intermediates.

intermediate of the *tsf* coat proteins has not yet been investigated. The *tsf* subunits are able to interact productively with scaffolding protein to produce procapsids that are altered from WT procapsids but still viable in phage biogenesis (C. M. Capen and C.M.T., unpublished results). When the *tsf* coat proteins are shifted to high-temperature either during folding or as folded subunits, I* becomes the predominant form and aggregates. GroEL/S intercede in the folding of the *tsf* mutants but not in the folding of WT coat protein even at high temperature (*39*). Again further experiments will be needed to determine how the I* intermediate interacts during aggregation.

Now that conditions have been found where it is possible to switch from the productive to nonproductive folding pathways with a simple increase in temperature, we can begin to understand how the conformation of folding intermediates controls the aggregation and folding pathways.

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REFERENCES

1. Anfinsen, C. B. (1973) *Science 181*, 223−230.
2. Ingram, V. M. (1957) *Nature 180*, 326−328.
3. Eaton, W. A., and Hofrichter, J. (1990) *Adv. Protein Chem. 40*, 63−279.
4. Dagliesh, R. (1997) *Nucleic Acids Res. 25*, 181−187
5. Dagliesh, R. (1998) *Nucleic Acids Res. 26*, 253−255.
6. Carrell, R. W., and Lomas, D. A. (1997) *Lancet 350*, 134−138.
7. Kelly, J. W. (1997) *Structure 5*, 595−600.
8. Helms, L. R., and Wetzel, R. (1996) *J. Mol. Biol. 267*, 4977−4983.
9. 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.
10. Marston, F. A. O. (1986) *Biochem. J. 240*, 1−12.
11. Mitraki, A., and King, J. (1989) *Biotechnology 7*, 690−699.
12. Teschke, C. M., and King, J. (1992) *Curr. Opin. Biotechnol. 3*, 468−473.
13. Betts, S. D., and King, J. (1998) *Protein Sci. 7*, 1516−1523.
14. Speed, M. A., Morshead, T., Wang, D. I. C., and King, J. (1997) *Protein Sci. 6*, 99−108.
15. Speed, M. A., Wang, D. I. C. and King, J. (1995) *Protein Sci. 4*, 900−908.
16. Speed, M. A., Wang, D. I. C., and King, J. (1996) *Nature Biotechnol. 14*, 1283−1287.
17. Mitraki, A., Danner, M., King, J., and Seckler, R. (1993) *J. Biol. Chem. 268*, 20071−20075.
18. Goldenberg, D., and King, J. (1981) *J. Mol. Biol. 145*, 633−651.
19. Chrunyk, B. A., Evans, J., Lillquist, J., Young, P., and Wetzel, R. (1993) *J. Biol. Chem. 268*, 18053−18061.
20. Brems, D. N. (1988) *Biochemistry 27*, 4541−4546.
21. Ranson N. A., White H. E., and Saibil H. R. (1998) *Biochem. J. 333*, 233−242.
22. Ewalt, K. L., Hendrick, J. P., Houry, W. A., Hartl, F.-U. (1997) *Cell 90*, 491−500.
23. Lorimer, G. H. (1996) *FASEB. J. 10*, 5−9.
24. Botstein, D., Waddell, C. H., and King, J. (l973) *J. Mol. Biol. 80*, 669−695.
25. Eppler, K., Wykoff, E., Goates, J., Parr, R., and Casjens, S. (1991) *Virology 183*, 519−538.
26. King, J., Lenk, E. V., and Botstein, D. (l973) *J. Mol. Biol. 80*, 697−73l.
27. King, J., and Casjens, S. (l974) *Nature 25l*, 112−119.
28. Fuller, M. T., and King, J. (l980) *Biophys. J. 32*, 38l−40l.
29. Prevelige, P. E., Thomas, D., and King, J. (1988) *J. Mol. Biol. 202*, 743−757.
30. Thuman-Commike, P. A., Greene, B., Jakana, J., Prasad, B. V. V., King, J., Prevelige, P. E., Jr., and Chiu, W. (1996) *J. Mol. Biol. 260*, 85−98.
31. Prevelige, P. E., Thomas D., and King, J. (1993) *Biophys. J. 64*, 824−835.
32. Thomas, D., and Prevelige, P. E., Jr. (1991) *Virology 182*, 673−681.
33. Bazinet, C., and King, J. (1985) *Annu. Rev. Microbiol. 39*, 109−129.
34. Earnshaw, W., and Casjens, S. (1980) *Cell 2l*, 3l9−331.
35. Prasad, B. V. V., Prevelige, P. E., Marieta, E., Chen, R. O., Thomas, D., King, J., and Chiu, W. (1993) *J. Mol. Biol. 231*, 65−74.
36. Gordon, C. L., and King, J. (1993) *J. Biol. Chem. 268*, 9358−9368.
37. Gordon, C. L., and King, J. (1994) *Genetics 136*, 427−438.
38. Gordon, C. L., Sather, S. K., Casjens, S., and King, J. (1994) *J. Biol. Chem. 269*, 27941−27951.
39. Nakonechny, W. S., and Teschke, C. M. (1998) *J. Biol. Chem. 273*, 27236−27244.
40. Teschke, C. M., and King, J. (1993) *Biochemistry 32*, 10839−10847.
41. Teschke, C. M., and King, J. (1995) *Biochemistry 34*, 6815−6826.
42. Galisteo, M. L., Gordon, C. L., and King, J. (1995) *J. Biol. Chem. 270*, 16595−16601.
43. Teschke, C. M., King, J., and Prevelige, P. E., Jr. (1993) *Biochemistry 32*, 10658−10665.

44. Andrews, A. T. (1986) in *Electrophoresis: Theory, techniques and Biochemical and Clinical Application* (Peacocke, A. R., and Harrington, W. F., Eds.) pp 5−78, Oxford University Press, New York.
45. Farris, F. J., Weber, G., Chiang, C. C., and Paul, I. C. (1978) *J. Am. Chem. Soc. 100*, 4469−4474.
46. Shi, L., Palleros, D. R., and Fink, A. L. (1994) *Biochemistry 33*, 7536−7546.
47. Secnik, J., Gelfand, C. A., and Jentoft, J. E. (1992) *Biochemistry 31*, 2982−2988.
48. Rabilloud, T., Carpentier, G., and Tarroux, P. (1988) *Electrophoresis 9*, 288−291.
49. Prevelige, P. E., Thomas, D. H., Aubrey, K. L., Towse, S. A., and Thomas, G. J. (1993) *Biochemistry 32*, 337−343.
50. Parker, M. H., and Prevelige, P. E. (1998) *Virology 250*, 337−349.
51. Fuller, M. T., and King, J. (l982) *J. Mol. Biol. 156*, 633−665.
52. Teschke, C. M., and Fong, D. G. (1996) *Biochemistry 47*, 14831−14840.
53. Betts, S. D., Hasse-Pettingell, C., and King, J. A. (1997) *Adv. Protein Chem. 50*, 243−264.
54. Freidfelder, D. (1982) *Physical Biochemistry. Applications to Biochemistry and Molecular Biology*, 2nd ed., pp 662−664, W. H. Freeman and Co., San Francisco, CA.
55. Wetzel, R. (1997) *Adv. Protein Chem. 50*, 183−242.
56. Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, Plenum, New York.

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