Alleviation of a defect in protein folding by increasing the rate of subunit assembly

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Running title: Folding suppressor increases aggregation and assembly rates

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Abbreviations: tsf, temperature-sensitive-folding; su, suppressor; WT, wild-type; U, unfolded coat protein; [I₁] or [I₂], coat protein folding intermediates; [I*], off-pathway intermediate; N, coat protein monomeric subunit.
Summary

Understanding the nature of protein grammar is critical because amino acid substitutions in some proteins cause misfolding and aggregation of the mutant protein resulting in a disease state. Amino acid substitutions in phage P22 coat protein, known as temperature-sensitive-folding (tsf) mutations, cause folding defects that result in aggregation at high temperatures. We have isolated global suppressor (su) amino acid substitutions that alleviate the tsf phenotype in coat protein (Aramli, L. A. & Teschke, C. M. (1999) J. Biol. Chem. 274, 22217-22224) (1). Unexpectedly, we found that a global su amino acid substitution in tsf coat proteins made aggregation worse and that the tsf phenotype was suppressed by increasing the rate of subunit assembly, thereby decreasing the concentration of aggregation-prone folding intermediates.
Introduction

The primary amino acid sequence of a polypeptide encodes all the information necessary for folding and assembly pathways, as well as the native three-dimensional structure (2). Substitutions and deletions in the amino acid sequence of a protein can have significant impact on the ability of a protein to fold or assemble properly. Depending on the protein, such changes in the amino acid sequence can lead to protein misfolding, mislocalization due to misfolding or aggregation (3,4). Amino acid substitutions in p53 lead to a misfolding problem compromising the function of the protein, resulting in cancer (5). Further, there are the diseases that result from mislocalization due to protein misfolding. For example, in α1-antitrypsin deficiency, a single amino acid substitution results in misfolding of α1-antitrypsin leading to the accumulation of long chain polymers within the hepatocyte. This leads to a reduction of the plasma concentrations of α-antitrypsin and predisposes individuals to emphysema and liver disease (6). Therefore, understanding the nature of protein grammar is of paramount interest.

While the process of protein folding is still not completely understood, it is known that larger proteins often have identifiable folding intermediates. These folding intermediates may interact inappropriately before reaching the native state. In fact, protein misfolding is a common problem faced by biotechnology companies that harvest proteins of commercial interest using recombinant DNA technologies in heterologous hosts (7-11). Often these proteins have problems with inclusion body formation, thereby decreasing the yield of pharmaceutically important products. Single amino acid substitutions can affect the folding pathway by shifting the folding from the productive pathway to off-pathway aggregation. For example, the amino acid substitutions in transthyretin causes a shift in the equilibrium between the native state and an aggregation-prone unfolding intermediate resulting in amyloid formation. Individuals with
any of the fifty known amino acid substitutions in transthyretin are predisposed to familial amyloidosis (12-15). While during the folding of P22 tailspike proteins with temperature-sensitive-folding (tsf) amino acid substitutions, a folding intermediate is aggregation prone at high temperatures (16-19). Other proteins such as interleukin-1ß (20) and bovine growth hormone (21) have similar tendencies to aggregate.

We use coat protein of bacteriophage P22 as a model system to study the processes of folding and assembly in vivo and in vitro (22). P22 is a dsDNA bacteriophage of Salmonella typhimurium. The T=7 icosahedral capsid is composed primarily of 420 coat protein subunits, each of which is a 47 kDa polypeptide of 430 amino acids. During the process of assembly, the monomeric coat protein subunits interact with 150-300 molecules of scaffolding protein (33 kDa) in a nucleation-dependent reaction to produce the procapsid, a precursor of the mature capsid. The nucleation-limited assembly reaction occurs by the addition of the monomeric coat protein subunits to the growing edge of the partially formed procapsid (23). Once the procapsid has assembled, the scaffolding protein exits through the holes present in the procapsid lattice while the DNA is actively packaged through the portal vertex. During this process there is an expansion of the capsid lattice into the mature capsid, which is characterized by a 15% increase in diameter, a change in shape from the spherical to icosahedral and the partial closing of holes in the lattice (24-26).

Previously, a group of amino acid substitutions in phage P22 coat protein were identified and characterized that result in a temperature-sensitive-folding (tsf) phenotype (27,28). In vivo, the tsf amino acid substitutions significantly reduce the yield of soluble coat protein at high temperatures because the newly synthesized tsf coat polypeptides aggregate to form inclusion bodies prior to reaching the mature assembly-competent state required for capsid assembly.
(27,29). As a consequence of the 

\( \text{tsf} \) defect, there is a decrease in the rate and yield of procapsid assembly both \textit{in vitro} and \textit{in vivo} (29,30). \textit{In vitro}, \text{tsf} coat protein monomers have altered secondary and tertiary structure, as well as increased surface hydrophobicity (30). Additionally, the \text{tsf} amino acid substitutions cause an increase in the rate of unfolding of coat protein; thereby increasing the concentrations of the folding intermediates (unpublished data). These \textit{in vitro} properties may explain the increased propensity of the folding intermediates to aggregate.

Based on the above observations, we present a model of folding and assembly of coat protein (Figure 1). It has been established that the folding of coat protein proceeds through at least two intermediates (\([\text{I}_1]\) and \([\text{I}_2]\)) to form an assembly-competent coat protein subunit (30). In \text{tsf} coat proteins, at high temperatures, there is an accumulation of an aggregation-prone, off-pathway intermediate (\([\text{I}^*]\)) from either \([\text{I}_1]\) or \([\text{I}_2]\) (30). The reaction producing an aggregate is one of two essentially irreversible reactions in the folding of coat protein. The second irreversible reaction is the assembly of procapsids from the monomeric subunit (N), which occurs upon the addition of scaffolding protein (31). The \text{tsf} coat proteins assemble with slower kinetics than wild-type (WT) coat protein, as a result of the \text{tsf} folding defect (30), and assembly does not correct the conformational defects of \text{tsf} coat proteins (32).

As a means of identifying additional amino acids that are critical for folding, second site suppressors (\(\text{su}\)) of \text{tsf} coat protein mutants were isolated (1). The most frequently isolated type of second site suppressors were global suppressors. Global suppressor amino acid substitutions are capable of alleviating the phenotype of multiple \text{tsf} mutants. These global suppressors were identified at positions 163, 166 and 170 in the amino acid sequence of coat protein (1), a region located in close proximity of a putative hinge domain of coat protein (33).
Here we examine the mechanism by which a global suppressor alleviates the tsf phenotype. Unexpectedly, we found that the presence of the global suppressor amino acid substitution T166I in the tsf coat proteins S223F and F353L lead to an increase in aggregation. Further, we have determined that the increase in aggregation of the tsf:su coat proteins is not likely to be the result of a decrease in the thermostability of the tsf:su coat proteins relative to the tsf parents. However, by a novel mechanism, the tsf phenotype was suppressed by an increase in the rate and yield of subunit assembly.
Experimental Procedures

**Bacteria** --- The bacteria used for all the experiments were derivatives of *Salmonella typhimurium* LT2. The amber suppressor minus host DB7136 (*leuA414-am, hisC525-am*) and its amber suppressor plus derivative DB7155 (*leuA414-am, his C525-am, supE20-gln*) have been described previously (34).

**Bacteriophage** --- The P22 bacteriophage used in this study were WT in gene 5 which encodes for coat protein, carried the *tsf* nucleotide substitutions in gene 5 leading to amino acid substitutions S223F or F353L, or carried *tsf:su* mutations in gene 5 leading to S223F:T166I or F353L:T166I (1). All phage strains used in these experiments carried the c1-7 allele, which prevents lysogeny. The phage also carried amber mutations in gene 3 and 13, to prevent DNA packaging and cell lysis, respectively, in order to produce procapsids.

**Chemicals** --- Ultrapure guanidine hydrochloride (GuHCl), urea, and silver nitrate were purchased from Schwartz-Mann ICN. HGT Seakem agarose was purchased from American Bioanalytical. All other chemicals were reagent grade purchased from common sources.

**Media** --- Luria broth (LB) was prepared as described by Life Technologies, Inc. and was used for initial bacterial growth of *Salmonella typhimurium*, DB7155. Superbroth was prepared to support bacterial growth for procapsid isolation. Superbroth contains 32 grams tryptone, 20 grams yeast extract, 5 grams NaCl, and 5 ml 1 N NaOH per liter of water (35).

**Buffer** --- The buffer utilized in all of the experiments was 20 mM sodium phosphate, pH 7.6. For procapsid preparations and storage of the shell stocks, the buffer utilized was 50 mM Tris base, 25 mM NaCl, and 2 mM EDTA, adjusted to pH 7.6 with HCl.
**Purification of Coat Proteins** --- WT, S223F, S223F:T166I, F353L and F353L:T166I used in the following experiments were obtained from empty procapsid shell stocks that were prepared as previously described (31,32,36,37). Briefly, *Salmonella typhimurium* (DB7155) were grown in LB to 1 X 10^8 cells/ml at 30 °C and infected with various strains of bacteriophage P22 at a multiplicity of infection (MOI) of 0.075 to prepare a large phage stock. To determine whether reversions of the amber mutations or the tsf phenotype had occurred, the phage stocks were checked for reversion frequency and temperature-sensitivity. Next, *Salmonella typhimurium* (DB7136, an amber minus strain) were grown in Superbroth to 4 X 10^8 cells/ml at 28 °C with vigorous aeration and infected with bacteriophage P22 at a multiplicity of infection of 5. Since the phage carried amber mutations which prevent cell lysis and DNA packaging, the infected cells accumulated procapsids. After 5 hours the chilled cells were pelleted by centrifugation at 4 °C at 10,000 rpm using a GSA rotor in a Sorvall RC-5B Superspeed Centrifuge. The collected cells were suspended in a small volume of cold buffer. The cells were lysed by a freeze/thaw cycle and were treated with a final concentration of 0.1 M phenylmethylsulfonyl fluoride (PMSF), then with 50 µg/ml RNase and 50 µg/ml DNase. The procapsids were pelleted in a Ti60 rotor in a Beckman L7-65 at 45,000 RPM for 35 min at 4 °C. The procapsid pellet was resuspended in a small amount of buffer with fresh PMSF by shaking overnight at 4 °C. The procapsids were purified using a Sephacryl S-1000 (Pharmacia-LKB) column to remove smaller contaminating proteins and membranes. To prepare empty procapsid shells that are composed solely of coat protein, the scaffolding protein was removed from the procapsids by repeated extractions with 0.5 M GuHCl followed by centrifugation to pellet the shells. Shell stocks concentrations were determined by absorbance of the unfolded shell stocks in 6 M GuHCl using
an extinction coefficient of 0.957 ml mg\(^{-1}\) cm\(^{-1}\) at 280 nm (38). All purified empty procapsid shells were suspended in buffer and stored indefinitely at 8.5 mg/ml at 4 °C.

**Refolding of Coat Protein by Rapid Dilution** --- Empty procapsid shells were unfolded at 2 mg/ml in 6.75 M urea, 20 mM phosphate buffer at pH 7.6 for 30 minutes at room temperature. Refolding was initiated by rapid dilution with phosphate buffer to yield a final coat protein concentration of 0.1 mg/ml with 0.34 M residual urea.

**Refolding of Coat Protein by Dialysis** --- Empty procapsid shells were unfolded in urea as described above. The denatured samples were diluted to 1.2 mg/ml and 4.0 M urea and then refolded by dialysis in a microdialyzer (Gibco-BRL Life Technologies) at 4 °C at a rate of 0.75 ml/min with phosphate buffer. The refolded coat protein was collected when urea was no longer detected by refractometry. Samples were centrifuged in a microfuge for 2 min at 13,000 rpm at 4 °C. The protein concentration was determined by absorbance at 280 nm.

**Native Polyacrylamide Gel and Agarose Electrophoresis** --- Samples for native polyacrylamide gels were prepared by combining a portion of the protein with 3X native gel sample buffer (30% glycerol, 112 mM Tris and 120 mM glycine). The samples (0.4 µg) were loaded onto native 4.3% polyacrylamide stacking gel (pH 8.3) and 7.5% native polyacrylamide resolving gel (pH 9.5) (39). Native polyacrylamide gels were run at 10 mA constant current at 4 °C for ~1 hour. The bands on the native polyacrylamide gels were visualized by silver staining (40). Samples for the native agarose gel were prepared by combining a portion of protein with agarose gel sample buffer (40 mM Tris base, 1 mM EDTA, 20% sucrose, pH 8.2 with acetic acid) and ~ 6 µg was loaded onto 1.2% Seakem HGT agarose gel made with the same buffer without the sucrose and run at 50 V for 3.5 hours at room temperature (41). The agarose gels were stained with
Coomassie blue (10% acetic acid containing 0.03% Coomassie brilliant blue R-250 and 0.02% Coomassie brilliant blue G-250) overnight and destained (10% acetic acid and 10% isopropyl alcohol) over several days.

**Procapsid Assembly Reactions** --- To assemble coat protein into procapsids, refolded coat protein at a final concentration of 83 µg/ml was mixed with scaffolding protein at a final concentration of 160 µg/ml at 20 °C in a total volume of 100 µl in the SLM Aminco-Bowman 2 spectrofluorometer. The reaction was monitored by the increase in 90 ° light scattering at 500 nm with the bandpasses set to 4 nm. Samples from each reaction were run on 1.2 % agarose gels as described above. Another method to monitor the assembly reaction is to refold urea-denatured coat protein and urea-denatured scaffolding protein together until the urea was no longer detectable by refractometry (35). Coat protein and scaffolding protein were each unfolded at 2 mg/ml in 6.75 M urea. Protein concentrations for scaffolding protein were determined at 280 nm using an extinction coefficient of 0.48 ml mg⁻¹ cm⁻¹ (42). Samples were refolded together at 1 mg/ml in a microdialyzer at a flow rate of 0.75 ml/min at 20 °C and 30 °C until residual urea was no longer detected. Samples were prepared and run on both native polyacrylamide and agarose gels as described above.
Results

As a means of identifying additional amino acids that are critical for folding second site suppressors of P22 tsf coat protein mutants were isolated (1). The most frequently isolated group of second site suppressors were global suppressors at positions 163, 166 and 170 of the coat protein (1). Global suppressors have been identified in other proteins (43-45). For example, the global suppressors of the tsf mutants in P22 tailspike protein have been shown to alleviate folding defects by decreasing the rate of aggregation through stabilization of a folding intermediate (17,46-48). Here we examine the mechanism by which a global suppressor alleviates the tsf phenotype in coat protein. Coat proteins carrying the global suppressor substitution T166I in the tsf backgrounds were chosen for this study because the temperature-sensitive phenotype of S223F and F353L was alleviated by all three global suppressors. Additionally, T166I was the most frequently isolated global suppressor amino acid substitution and has proven capable of improving the folding and assembly of tsf coat proteins to WT levels (1).

Aggregation of tsf and tsf:su coat proteins during folding

Based on the results from the tailspike protein experiments, we believed it likely that the global suppressors of coat protein would also decrease the propensity of the protein to aggregate during folding. To determine the tendency of the tsf:su coat proteins to aggregate during folding, WT, S223F, S223F:T166I, F353L, and F353L:T166I coat proteins were first unfolded in denaturant. Refolding was initiated by rapid dilution at various temperatures. Aliquots were taken after refolding had been initiated and run on a native polyacrylamide gel (Figure 2). The band of highest mobility corresponds to the folded monomeric form while the aggregates are the ladder of bands of slower mobility (11,18,30,49). None of the coat protein samples aggregated
at 15 °C or below. The band of monomeric WT coat protein remained constant at all temperatures tested. As previously reported, a small amount of aggregation of WT coat protein was observed at 33 °C and above, but appeared to originate from a band of decreased mobility, suggesting that the aggregation was likely due to incorrectly or slowly folding polypeptides which did not run in the monomer position on the native gel (30). In contrast, the tsf mutant S223F began to substantially aggregate at 20 °C. Surprisingly, S223F:T166I demonstrated a significant increase in the rate of aggregation at 20 °C compared to its tsf parent, as seen by an increase in the intensity and number of bands of slower mobility present in the tsf: su coat protein (Figure 2). With increasing temperature, the intensity of the bands of slower mobility further increased and the intensity of the monomeric bands decreased in S223F:T166I over time when compared to its tsf parent, S223F. The increased tendency to aggregate when the su substitution T166I was present was also observed with F353L:T166I, though both F353L and F353L:T166I began to aggregate at a higher temperature than S223F and S223F:T166I. This is consistent with the in vivo phenotype of F353L (1,50). F353L began to aggregate at 33 °C, while F353L:T166I had increased bands of aggregation at the same temperature. Based on this experiment we conclude that the presence of the su substitution T166I unexpectedly increased the propensity for the tsf coat proteins to aggregate during folding.

**Aggregation of tsf and tsf: su coat proteins from the folded state**

One possible explanation for why the tsf: su coat proteins were more aggregation prone was that these monomers were less thermostable than the tsf coat protein monomers. Previously, the folded tsf coat proteins were shown to be about as stable as WT coat protein by differential scanning calorimetry and denaturation with pressure (36,51). However, recent equilibrium folding and unfolding experiments with the tsf coat protein mutants have indicated that the
proteins were generally less stable to denaturant than WT coat protein (unpublished data). Therefore, in order to determine the thermostability of the tsf: su coat proteins, we first refolded tsf and tsf: su coat proteins to their native conformation at 4 °C and then shifted samples to higher temperatures. The circular dichroism and tryptophan fluorescence spectra of the refolded tsf proteins were the same as previously published (30). The spectra of the tsf: su proteins were similar to their tsf parents and were therefore consistent with folded structure (data not shown). Aliquots were taken after the temperature shift-up and run on native polyacrylamide gels to monitor aggregation of the proteins (Figure 3). WT coat protein was resistant to aggregation at the various temperatures tested up to 39 °C, where aggregation begins, consistent with our previous work (30). S223F:T166I coat protein was only slightly more aggregation prone than the S223F coat protein. Similar results were obtained when comparing the thermostability of F353L and F353L:T166I, though they aggregated slightly less than S223F and S223F:T166I. Thus, it appears unlikely that the increase in aggregation of the tsf: su coat proteins was due to a decrease in the thermostability of the folded monomeric state.

Assembly of tsf and tsf: su coat proteins

Since the tsf: su coat proteins have an increased propensity to aggregate during folding, the su amino acid substitution must suppress the tsf phenotype at a step other than the [I₁, or I₂] ↔ [I*] transition (Figure 1). Because procapsid assembly is the other irreversible step in the folding pathway of coat protein and could potentially shift the folding equilibrium to the right by decreasing the concentration of N, we examined the effect of the su amino acid substitution on this reaction. Samples of WT, S223F, S223F:T166I, F353L and F353L:T166I coat proteins were refolded by dialysis at 4 °C to form monomeric coat protein. The refolded coat proteins were mixed with scaffolding protein at 20 °C. The assembly reaction was monitored by the increase
in light scattering at 500 nm (Figure 4). WT coat protein was assembly-competent and formed procapsids, as seen by the increase in light scattering (31). The *tsf* coat protein S223F was assembly-incompetent and F353L had low assembly activity. In contrast, addition of the *su* amino acid substitution in the *tsf* background dramatically increased both the rate and the yield of procapsid assembly. Because aggregates are large and can also scatter light, we confirmed that the increase in light scattering was the result of procapsid formation by running the assembly reactions on native agarose gels where coat protein was found to be in either procapsid form or as folded monomers (data not shown). Therefore, while the *su* amino acid substitutions increased the aggregation propensity of the *tsf:su* coat proteins during folding, once folded, procapsid assembly was enhanced.

*In vivo*, a P22 bacteriophage-infected cell would have scaffolding protein present during the folding of coat protein. To determine if the presence of scaffolding protein during folding would decrease the aggregation reaction by favoring subunit assembly, unfolded coat and unfolded scaffolding protein were mixed together at equal concentrations and dialyzed at various temperatures until urea was no longer detected (52). The samples were run on a native agarose gel to detect procapsids (Figure 5a). As expected, when WT coat protein was folded with WT scaffolding protein, subunit assembly occurred, yielding procapsids. Conversely, the *tsf* coat proteins, S223F and F353L, did not assemble into procapsids. The *tsf:su* coat proteins S223F:T166I and F353L:T166I formed procapsids at both 20 °C (Figure 5a) and 30 °C (data not shown), though there was a decrease in the yield of the procapsids at the higher temperature. Additionally, the same samples were run on native polyacrylamide gels to monitor aggregation (Figure 5b). We observed a significant decrease in the intensity of the bands corresponding to aggregates in the reaction where the *tsf:su* coat proteins were refolded with scaffolding protein as
compared to tsf:su coat protein refolded without scaffolding protein (Figure 5b). Thus, our experiments indicate that the presence of scaffolding protein during folding of the tsf:su coat proteins decreased their tendency for aggregation by increasing the yield and rate of procapsid assembly.
Discussion

Aggregation is a serious problem for both biotechnology utilizing recombinant DNA technologies in heterologous hosts (7-11), and for various proteins with amino acid substitutions such as transthyretin (12-15). Thus, learning to control aggregation is of the utmost importance. Here we have investigated the effect of a global suppressor amino acid substitution on the aggregation propensity of tsf coat proteins in an effort to determine the mechanism of suppression of the tsf folding defects. Surprisingly, we have found that the tendency to aggregate increases in the presence of the suppressor amino acid substitution, and that the suppression of the original tsf defect occurs by increasing the rate and yield of subunit assembly. This may have general implications in improving the folding and yield of multimeric proteins.

Global Suppressors

In addition to our global suppressors of tsf mutants in P22 coat protein (1), other second site suppressors have been found which alleviate an original folding defect. P22 tailspike protein is a well studied protein. A group of tsf mutants of P22 tailspike protein have been identified and characterized to be defective in folding and not stability (16,53,54). Analysis of the folding pathway revealed that aggregation of tailspike protein occurs by the association of a partially folded monomeric intermediate, rather than the native trimeric species. The thermolabile intermediate preferentially partitions onto the aggregation pathway as the temperature increases (55). In order to determine other positions in the amino acid sequence important to the folding process, second site suppressors of the tsf tailspike protein mutants were isolated (56). Two major global suppressor substitutions were found to alleviate the folding defects of multiple tsf mutants of tailspike protein (46). Structural resolution of a truncated version of tailspike protein revealed that the two global suppressors, V331A and A334V, were located in the parallel β-helix
domain and the associated “dorsal fin domain” of the fishlike structure. The global suppressors of the tsf mutants in tailspike protein decrease aggregation in vivo and in vitro (47). However, the global suppressor amino acid substitutions act by different mechanisms (48). The suppressor, V331A, alleviates the tsf folding defects by stabilizing the completely folded protein by reducing steric hindrance in the native state. The suppressor, A334V, alleviates the folding defects in a more complicated way. The A334V suppressor substitution accelerates unfolding at high temperature, thereby decreasing the stability of the trimeric tailspike protein. The A334V substitution also increases the stability of an early folding intermediate by improving hydrophobic, therefore improving the overall folding of the tailspike protein.

Examples of global suppressors of folding mutants of proteins other than phage proteins exist. For instance, variants of chloramphenicol acetyltransferase, a bacterial enzyme that confers resistance to the antibiotic chloramphenicol, have been isolated. These mutants quantitatively aggregate into cytoplasmic inclusion bodies, resulting in a lack of chloramphenicol resistance. Van der Schueren et al. (1998) (57) isolated second site suppressors of these variants of chloramphenicol acetyltransferase. The global suppressor, L145F, improved the thermostability of the protein and its ability to fold into a soluble, enzymatically active conformation. Similarly, temperature-sensitive mutants of the human receptor-like protein tyrosine phosphatase LAR have both stability and folding defects that result in the aggregation of the protein. The presence of a suppressor amino acid substitution in LAR decreased the aggregation propensity as seen by an increase in production of the properly folded protein. The decrease in aggregation occurs, at least in part, by an increase in stabilization of mutant protein (43). A second amino acid substitution, M182T, is often found along with substitutions in TEM-1 β-lactamase that confer increased resistance to antibiotics (44). The M182T substitution was
shown to decrease the stability of the protein to denaturant but increased the solubility of the double mutant (58). The exact mechanism by which M182T functions to decrease aggregation is still to be established. Here the suppressor substitution has been proposed to either inhibit aggregation by changing the conformation of an aggregation-prone intermediate or to alter the folding mechanism in a way to kinetically avoid aggregation (58).

**Mechanisms for suppression of folding defects**

Together, the different suppressor amino acid substitutions of the various mutants of P22 tailspike protein, chloroamphenicol acetyltransferase, and human receptor-like protein tyrosine phosphatase LAR have been shown to correct the folding problem by stabilizing either the native conformation of the protein or a folding intermediate. These examples are in contrast to the novel mechanism we have elucidated for tsf:su coat protein mutants. Based on our experiments, it is clear that there is actually an increased propensity of the tsf:su coat protein mutants to aggregate during folding. However, the tsf:su mutants coat proteins have improved subunit assembly capability, thereby suppressing the original tsf phenotype. We believe that this method could be used generally to improve the folding yield of multimeric proteins.
Acknowledgements

This work was supported by NIH grant GM53567. We would also like to thank Carole Capen, Shannon Doyle, Dr. Todd Garabedian, Walter Nakonechny and Mark Tardie for their helpful comments and discussions.
References


Figure Legends

Figure 1. A model of the folding and assembly of coat protein. In our model, U represents the unfolded coat protein, \([I_1]\) and \([I_2]\) represent the intermediates, \([I^*]\) an off-pathway intermediate that is aggregation-prone, and N represents the folded state of coat protein monomers. N assembles into procapsids in association with scaffolding protein. The irreversible reactions are indicated with the heavy uni-directional arrows while the reversible reactions are shown with the lighter, bi-directional arrows. The tsf amino acid substitutions increase the rate of unfolding from N to \([I_2]\) (unpublished data).

Figure 2. Aggregation of WT, tsf and tsf:su coat proteins during folding. Samples of WT, S223F, S223F:T166I, F353L and F353L:T166I were unfolded in urea as described in the Experimental Procedures. Refolding was initiated by rapid dilution at various temperatures (4 °C to 36 °C). Aliquots were taken at 0.3, 3.5, 7.0, 12.0, and 15.0 minutes after the refolding reaction was initiated and placed in native gel sample and held on ice. The samples were run on native polyacrylamide gel as described in the Experimental Procedures (30,39). Bands were visualized by silver staining (40). The band with the highest mobility corresponds to the folded monomeric form of the various coat protein species (solid circle). The bands of slower mobility are the aggregates (bracket).

Figure 3. Aggregation propensity of WT, tsf and tsf:su coat proteins from the native state. WT, S223F, S223F:T166I, F353L and F353L:T166I coat protein were unfolded in urea as described in the Experimental Procedures. The samples were refolded by dialysis at 4 °C as described in the Experimental Procedures. Temperature shift-up experiments were performed at 4 °C to 39 °C. Aliquots were taken at 0, 3.5, 7.0, 12.0 and 15.0 minutes after the sample was
placed in circulating water bath at a specific temperature and immediately placed in native gel sample buffer and held on ice. Samples were run on a native polyacrylamide gel as described in the Experimental Procedures and silver stained. The bands of highest mobility correspond to the folded monomeric form of the various coat protein species (solid circle). The bands of slower mobility are the aggregates (bracket) (30).

**Figure 4. Assembly of WT, tsf and tsf:su coat proteins.** WT, S223F, S223F:T166I, F353L and F353L:T166I coat proteins were unfolded in urea as described in the Experimental Procedures. Samples were refolded by dialysis overnight at 4 °C at a flow rate of 0.3 ml/min. Refolded coat protein and refolded scaffolding protein were mixed together and monitored by light scattering at 20 °C as described in the Experimental Procedures (30).

**Figure 5. Assembly by refolding of coat protein with scaffolding protein.** WT, S223F, S223F:T166I, F353L and F353L:T166I coat proteins and scaffolding protein were unfolded in urea as described in the Experimental Procedures. After dilution, unfolded coat and unfolded scaffolding protein were refolded together by dialysis at various temperatures (20 °C and 33 °C) as described in Experimental Procedures. A. Samples were run on an agarose gel as described in the Experimental Procedures. Bands were visualized by Coomassie staining. B. Samples were run on a native polyacrylamide gel as described in the Experimental Procedures and silver stained. The bands of highest mobility correspond to the folded monomeric form of the various coat protein species. The band of lowest mobility, not entering the gel corresponds to the procapsid. The bands of slower mobility are the aggregates (30).
Figure 1

U → [I] ↔ [I₂] ↔ N → Procapsid

Scaffolding protein

[I*]

Aggregate
Figure 2
Figure 4

Light Scattering at 500 nm

Time (sec)

WT
S223F
S223F:T166I
F353L
F353L:T166I
S223F
Figure 5

A

assembly reactions at 20°C

Folded coat protein

Procapsid

B

refolded coat protein at 20°C

assembly reactions at 20°C

Procapsid

Aggregate

Folded coat protein
Alleviation of a defect in protein folding by increasing the rate of subunit assembly
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J. Biol. Chem. published online April 13, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M101759200

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