Molecular Microbiology (2004) **54**(4), 1036–1050 doi:10.1111/j.1365-2958.2004.04326.x

A second-site suppressor of a folding defect functions via interactions with a chaperone network to improve folding and assembly *in vivo*

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Summary

Single amino acid substitutions in a protein can cause misfolding and aggregation to occur. Protein misfolding can be rescued by second-site amino acid substitutions called suppressor substitutions (*su*), commonly through stabilizing the native state of the protein or by increasing the rate of folding. Here we report evidence that *su* substitutions that rescue bacteriophage P22 temperature-sensitive-folding (*tsf*) coat protein variants function in a novel way. The ability of *tsf:su* coat proteins to fold and assemble under a variety of cellular conditions was determined by monitoring levels of phage production. The *tsf:su* coat proteins were found to more effectively utilize P22 scaffolding protein, an assembly chaperone, as compared with their *tsf* parents. Phage-infected cells were radioactively labelled to quantify the associations between coat protein variants and folding and assembly chaperones. Phage carrying the *tsf:su* coat proteins induced more GroEL and GroES, and increased formation of protein:chaperone complexes as compared with their *tsf* parents. We propose that the *su* substitutions result in coat proteins that are more assembly competent *in vivo* because of a chaperone-driven kinetic partitioning between aggregation-prone intermediates and the final assembled state. Through more proficient use of this chaperone network, the *su* substitutions exhibit a novel means of suppression of a folding defect.

Introduction

It is widely recognized that the amino acid sequence determines the fold for a particular protein (Anfinsen, 1973). However, single amino acid substitutions can cause protein folding to go awry and aggregation can occur as a result. For example, human diseases such as cystic fibrosis (Thomas *et al*., 1992), Alzheimer's disease (Castano *et al*., 1995) and osteogenesis imperfecta (Bachinger *et al*., 1993) are all caused by the misfolding of proteins because of single amino acid substitutions. Single amino acid substitutions that cause a protein to aggregate highlight sites within the protein that are critical for proper folding and stability of the molecule. In addition, some second-site amino acid substitutions, termed suppressor substitutions, revert the misfolding caused by the original substitution. Suppressor substitutions highlight additional sites in the protein important for folding.

We chose the coat protein of bacteriophage P22, a double-stranded DNA (dsDNA) phage that infects *Salmonella typhimurium*, to study protein folding and assembly as there are 18 single amino acid substitutions in coat protein that cause a temperature-sensitive-folding (*tsf*) phenotype (Gordon and King, 1993). Previously, we isolated several second-site suppressor substitutions that revert the *tsf* phenotype back to wild type (WT). Some suppressor substitutions, called global suppressors (*su*), are able to revert the phenotype of several *tsf* substitutions. *In vitro*, these global suppressors make the aggregation of coat protein worse, but improve the ability of the *tsf* coat proteins to assemble into procapsids in the presence of scaffolding protein (Aramli and Teschke, 1999; 2001).

P22 coat protein is comprised of 429 amino acids for a monomer molecular weight of 47 kDa (Eppler *et al*., 1991). During assembly, 420 coat protein monomers coassemble with 150–300 scaffolding proteins (Casjens and King, 1974), pilot proteins (Bryant and King, 1984) and the portal complex (Bazinet *et al*., 1988) into a procapsid, which is the precursor to the mature phage. Once the procapsid is assembled, dsDNA packaging occurs and the scaffolding protein exits (Casjens and King, 1974; Earnshaw *et al*., 1976). A concomitant conformational change converts the round appearing procapsid to the classic icosahedral shape of many viruses, and expands the volume of the head by ≈10% (Prasad *et al*., 1993; Zhang *et al*., 2000). The phage head is stabilized by plug proteins, and ultimately tailspike proteins are added to

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form the infectious phage particle (Strauss and King, 1984).

Scaffolding protein, an assembly chaperone, is essential for correct phage P22 production. P22 normally adopts T = 7 icosahedral symmetry (Casjens, 1979); however, when scaffolding protein is eliminated or mutated, T = 7, T = 4 and aberrant spiral structures are observed by electron microscopy (Earnshaw and King, 1978; Thuman-Commike *et al*., 1998). Scaffolding protein also plays a catalytic role in assembly by allowing coat protein to associate with scaffolding protein at the growing edge of the virus shell (Prevelige *et al*., 1993). Thus, scaffolding protein:coat protein interactions have an important role in P22 assembly. Scaffolding proteins are required for proper assembly of many viruses, including the eukaryotic herpesvirus and adenovirus, as well as other dsDNA phage (Dokland, 1999) and may play several roles during virus assembly. Examples of these roles include forming a core of scaffolding proteins around which coat proteins correctly oligomerize; exclusion of other proteins from assembly (Earnshaw and Casjens, 1980); aiding in DNA packaging (King and Chiu, 1997); and incorporation of portal proteins into the virus head (Murialdo and Becker, 1978).

In addition to assembly chaperones, folding chaperones often play a role in virus production (Sullivan and Pipas, 2001). Chaperones, such as the major bacterial chaperone complex GroEL and GroES, assist the folding of many proteins, especially those prone to misfolding and aggregation (Sigler *et al*., 1998; Ellis, 2001; Thirumalai and Lorimer, 2001; Horwich, 2002). For bacteriophage P22, WT coat protein is not a substrate for GroEL and GroES (Nakonechny and Teschke, 1998). However, both the *tsf* and the global *su* substitutions cause coat protein to become a substrate for GroEL and GroES (Gordon *et al*., 1994; Aramli and Teschke, 1999). Both the *tsf* and *tsf:su* coat proteins are more aggregation-prone than WT coat protein. Furthermore, the *tsf*:*su* coat proteins are more aggregation-prone than the *tsf* coat proteins. The increase in aggregation propensity is a result of the highly populated folding intermediate state of the *tsf:su* coat proteins (Aramli and Teschke, 2001; Anderson and Teschke, 2003; Doyle *et al*., 2003; 2004). However, the presence of scaffolding protein minimizes aggregation by enhancing assembly *in vitro* for the *tsf:su* mutants (Aramli and Teschke, 2001).

Based on our *in vitro* data, we hypothesized that by increasing the rate of assembly, the global *su* substitutions decreased the concentration of aggregation-prone intermediates. We have shown that the *tsf* and *tsf*:*su* coat proteins flicker between a folded and an intermediate state and can be trapped either as an intermediate via aggregation or as a folded protein via capsid assembly (Aramli and Teschke, 2001; Doyle *et al*., 2003; 2004). In addition, we have shown that GroEL and GroES assist in the proper folding of *tsf* and *tsf:su* coat proteins (Nakonechny and Teschke, 1998; Aramli and Teschke, 1999; Doyle *et al*., 2003). We envision this as a kinetic competition between assembly and aggregation pathways. Here we investigate the *in vivo* folding and assembly of coat proteins carrying both a *tsf* and a *su* substitution to determine the molecular mechanism for suppression. We find that GroEL, GroES and scaffolding protein function in concert as coat protein chaperones *in vivo*. GroEL and GroES act to minimize coat protein aggregation by promoting correct folding from the increased pool of folding intermediates of the *tsf:su* coat proteins, whereas the addition of scaffolding protein propels the reaction towards procapsid formation from the increased pool of correctly folded coat proteins. Thus, we propose that second-site suppressor substitutions function to alleviate the *tsf* phenotype of coat proteins via kinetic partitioning through this chaperone network.

Results

In this study, we focus on the role of our global second-site suppressor substitutions in alleviating the folding defects caused by *tsf* substitutions in coat protein. The global suppressors were isolated in a search for second-site amino acid substitutions that reverted the *tsf* phenotype to that of WT coat protein while retaining the original *tsf* substitutions (Aramli and Teschke, 1999). Three global *su* substitutions were isolated repeatedly and were the most frequent of the substitutions isolated: D163G, T166I and F170L (Aramli and Teschke, 1999). Of these, T166I was the most often isolated and so was chosen for further study in conjunction with the *tsf* substitutions, S223F and F353L. These *tsf* substitutions were chosen from the 18 *tsf* substitutions because all three global suppressors rescued both S223F and F353L. In addition, S223F:T166I and F353L:T166I are our most thoroughly characterized *tsf*:*su* coat proteins from *in vitro* experiments (Aramli and Teschke, 1999; 2001; Doyle *et al*., 2004). Here we test the role of scaffolding protein and GroEL/ES in the folding and assembly of *tsf* and *tsf*:*su* coat proteins *in vivo*.

The effect of scaffolding protein and GroEL and GroES levels on coat protein folding and assembly

As phage production is sensitive to the condition of coat protein (i.e. misfolded coat protein aggregates and cannot produce infectious phage), burst experiments, which directly count the number of phage produced per cell, correlate production of functional coat protein under different conditions. To test whether scaffolding protein and GroEL/ES aided in suppression of the folding defect by the *tsf*:*su* coat proteins, phage bursts were compared

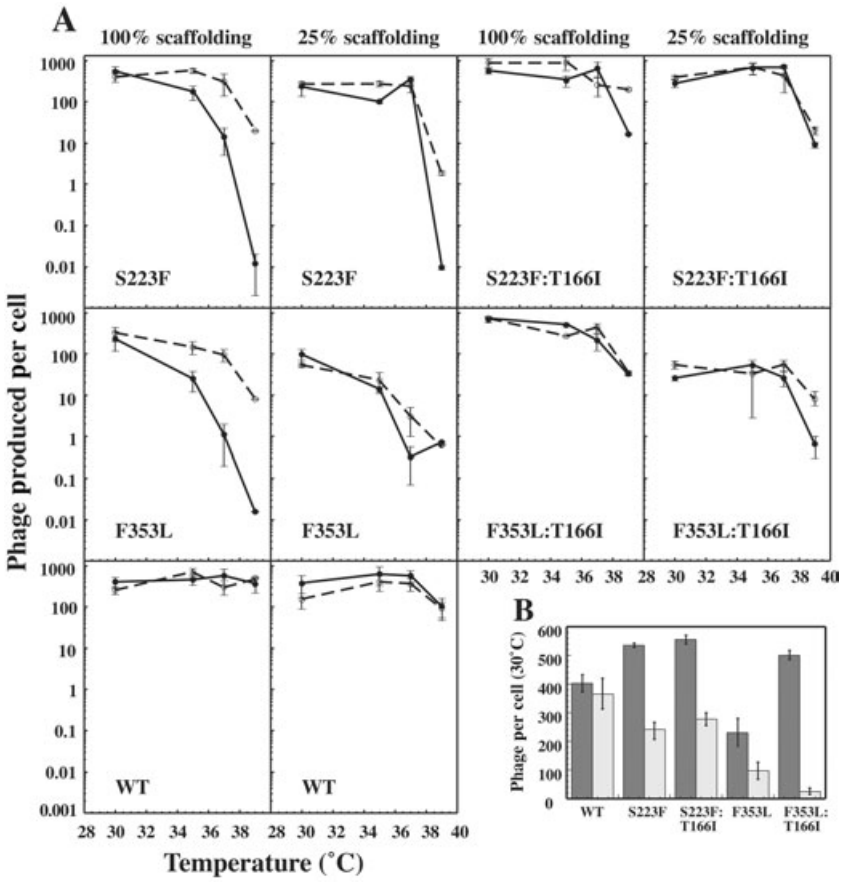

Fig. 1. Burst size of phage with *tsf*, *tsf:su* or WT coat protein.
A. Phage that were WT, or had *tsf* or *tsf:su* substitutions in coat protein were grown on normal cells (solid line), or on cells that overproduce GroEL and GroES (dotted line). The burst size is the number of phage produced per host cell at each experimental temperature and condition. The data were corrected for the number of cells infected. Error bars represent the standard deviation from three data sets. The data are plotted on a log scale; therefore there is a non-symmetrical appearance in the error bars.
B. The number of phage produced per cell for the various mutants at 30°C for easier comparison. Dark grey bars represent normal cellular conditions and light grey bars represent the number of phage produced per cell with 25% scaffolding protein. Error bars represent the standard deviation from three data sets.

under normal cellular conditions, and when the levels of both GroEL/ES and P22 scaffolding protein were varied. Phage that were either WT or carrying *tsf* or *tsf:su* substitutions in coat protein were grown on either WT cells or cells that contain a plasmid which overexpresses GroEL and GroES by approximately nine- to 10-fold (Gordon *et al*., 1994). The burst experiments were also performed when only 25% of normal amounts of scaffolding protein were produced, by a method detailed in *Experimental procedures*.

Wild-type coat protein does not readily aggregate at higher temperatures, nor does it require GroEL/ES for folding and assembly (Nakonechny and Teschke, 1998; Aramli and Teschke, 1999). However, WT coat protein does require scaffolding protein to direct proper phage assembly (Casjens and King, 1974; Casjens *et al*., 1985). Therefore, we performed burst experiments with WT phage to determine the effect of decreased levels of scaffolding protein at various temperatures (Fig. 1A and B). The number of WT phage produced per cell under normal cellular conditions was consistent with previously published results (Aramli and Teschke, 1999). Surprisingly, results from the burst experiments showed the production of WT phage was essentially unaffected by a 75% decrease in scaffolding protein (Fig. 1A).

Phage carrying *tsf* coat proteins showed decreased phage production as a function of temperature, coupled with rescue from the overexpression of GroEL/ES as previously shown (Fig. 1A) (Gordon *et al*., 1994). Interestingly, when scaffolding protein levels were decreased by 75%, the number of phage produced per cell decreased for all *tsf* coat proteins, in contrast to WT coat protein (Fig. 1A and B). Overexpressed GroEL and GroES were not able to significantly rescue *tsf* phage production at elevated temperatures when scaffolding protein levels were decreased (Fig. 1A), suggesting that scaffolding protein and GroEL and GroES may act in concert.

The *su* substitutions were selected by increased phage production at elevated temperatures as compared with their *tsf* parents (Aramli and Teschke, 2001). Indeed, we find that at permissive conditions, where there is very little competing aggregation, both *tsf:su* mutant coat proteins produce slightly more phage per cell than phage carrying WT coat protein (Fig. 1A). In addition, at all temperatures, phage with either *tsf:su* coat protein, S223F:T166I or F353L:T166I, showed an increase in phage production as compared with each *tsf* parent, consistent with the selection process. Under conditions of decreased scaffolding protein, the *tsf:su* coat protein mutants showed a reduc-

tion in the amount of phage produced even at permissive temperatures (Fig. 1B).

Decreasing scaffolding protein levels has a clear effect on the assembly of all of the coat protein variants *in vivo*. Additionally, the folding chaperones, GroEL and GroES, are required at elevated temperatures by the coat protein variants (Gordon *et al*., 1994; Nakonechny and Teschke, 1998; Aramli and Teschke, 1999). The use of chaperones is a logical mechanism by which aggregation during folding can be decreased. Our burst data, described above, suggest that the *su* substitution in coat protein functions by altering the kinetic partitioning between aggregation of folding intermediates and phage assembly through the use of both assembly and folding chaperones, even at permissive temperatures where aggregation is minimal. Therefore, in the following experiments we decided to study *tsf:su* coat protein interactions with scaffolding protein and GroEL/ES under permissive conditions.

Tsf:su *coat proteins interact more efficiently with scaffolding protein than their* tsf *parents*

As WT, *tsf* and *tsf:su* coat proteins demonstrated different levels of phage production with decreased levels of scaffolding protein, we reasoned that assembly of procapsids from WT and mutant coat proteins might have different requirements for scaffolding protein. To further characterize the role of scaffolding protein in assembly, we determined the ratio of scaffolding protein to coat protein contained in procapsids assembled *in vivo*.

Phage-infected cells were pulse labelled with [^{35}S]-methionine and cysteine at 30°C. This temperature was chosen to minimize aggregation and maximize assembly to investigate the role of chaperones in the suppression of coat protein folding defects under conditions where the chaperone network is fully functioning to alleviate aggregation. The radiolabelled proteins were separated by sucrose gradient sedimentation and fractions were analysed by SDS-PAGE.

Sucrose gradient sedimentation can easily separate coat protein monomers, procapsids and coat protein aggregates (Gordon and King, 1993; Doyle *et al*., 2003). Procapsids and large aggregates are found in the last fraction of the gradient under the conditions used in these experiments. We determined that less than 5% of coat protein from WT or any mutant was aggregated under these conditions using pellet/supernatant separations of the original material applied to the gradient (data not shown). Therefore, coat protein in the last fraction of the gradient was present as assembled structures, not aggregates. These assembled structures were procapsids, and not mature phage, because of the absence of tailspike protein in these samples as tailspike protein is added only after DNA packaging (Israel *et al*., 1967; King *et al*., 1973). The amount of radiolabel incorporated into both coat protein and scaffolding protein in the procapsids was quantified by densitometry. The ratio of coat protein to scaffolding protein was determined (Fig. 2). As 420 coat protein monomers are required to make a procapsid, any variation in the ratio of coat protein to scaffolding protein between phage strains must result from differences in the number of scaffolding proteins required for assembly.

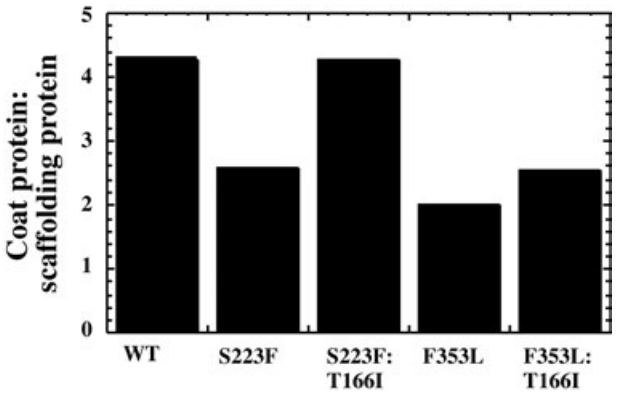

Fig. 2. Procapsid composition with WT, *tsf* and *tsf:su* coat proteins. Pulse-chase experiments, described in *Experimental procedures*, were performed on cells infected with phage that carried *tsf*, *tsf:su* or WT coat protein at 30°C. Fractions from 5% to 20% sucrose gradients were run on SDS gels. The bottom fraction contained the procapsids. The protein bands corresponding to coat and scaffolding proteins in the procapsids were quantified using densitometry. The ratio of coat protein to scaffolding protein present in procapsids for each mutant was calculated. A larger number indicates that less scaffolding protein was required to form a procapsid. These data are a representative sample. This experiment was repeated using different strains of bacteria and yielded similar results; however, this particular set was chosen for presentation because it represented the experiment carried out under the most physiological conditions.

For WT procapsids, a very high ratio of coat protein to scaffolding protein was observed and was similar compared with previously published reports (Fig. 2) (King *et al*., 1978). Thus, about one scaffolding protein molecule was incorporated for every four WT coat proteins in procapsids. This correlates well with our observations from the burst experiments. When 75% scaffolding was removed, WT phage production was relatively unaffected, indicating that more scaffolding protein was produced than necessary in a WT infection. Thus, WT coat protein interacts extremely efficiently with scaffolding protein *in vivo*.

The procapsids of both *tsf:su* mutants contained less scaffolding protein than their *tsf* parents. The *tsf:su* procapsids had one scaffolding protein for every 2.5–4 coat proteins, whereas the *tsf* mutants required about one scaffolding protein for every two coat proteins to assemble a procapsid (Fig. 2). This variation in the amount of scaffolding protein required for assembly of the coat protein variants could result from differences in affinity, assembly rate

or scaffolding protein exit from procapsids during recycling. These procapsid compositions correlate well with the burst experiments where decreasing scaffolding protein had a negative effect on both *tsf* and *tsf*:*su* mutants even at permissive temperatures (Fig. 1B), although the *tsf*:*su* coat protein variants did not require as much scaffolding protein to assemble a procapsid as their *tsf* parents. The *su* substitution is proposed to function in part as a suppressor of the *tsf* folding defect by causing the *tsf*:*su* coat proteins to more effectively interact with scaffolding protein.

Tsf:su *coat proteins produce more procapsids than their* tsf *parents when scaffolding protein is readily available* in vivo

In vitro assembly reactions showed that the *tsf:su* coat proteins are more assembly competent than their *tsf* parents when scaffolding protein is in excess (Aramli and Teschke, 2001). To further characterize the role of scaffolding protein in suppression of the *tsf* folding defect, we tested whether the *tsf:su* coat proteins were more assembly competent than their *tsf* parents when scaffolding protein is in excess *in vivo*.

Pulse-chase experiments using phage carrying WT, *tsf* or *tsf*:*su* coat proteins were performed as above, again at 30°C to minimize aggregation and maximize assembly. For this experiment, the phage also carried amber mutations in genes 3 and 13, which prevent DNA packaging and cell lysis respectively (Botstein *et al*., 1973; Casjens and King, 1974). In these infections, procapsids are assembled normally but DNA packaging cannot occur, scaffolding protein is not recycled, and because of its autoregulation, the amount of scaffolding protein produced is in excess (Botstein *et al*., 1973; King *et al*., 1978). This allows us to follow procapsid synthesis where scaffolding protein levels are never limiting. Therefore, any differences in ability to assemble must result from the amino acid substitutions in coat protein.

The radiolabelled cell lysates were run on agarose gels to separate the procapsids from other proteins. The amount of radiolabelled proteins incorporated into procapsids was quantified by densitometry, represented as pixels (Fig. 3). The *tsf* coat proteins were quite defective for assembly, even at 30°C. From the burst experiments, we know that the *tsf* coat proteins are able to form capsids, but here we see that their assembly is slow. Conversely, WT coat protein and the *tsf*:*su* coat proteins were able to efficiently assemble into procapsids. Interestingly, both *tsf:su* coat proteins also exhibited more rapid procapsid formation than WT coat protein. This correlates well with the burst experiments where both *tsf:su* coat proteins produced slightly more phage than WT coat protein at 30°C. In these experiments scaffolding protein is not limiting; therefore, the observed changes again indicate that the *tsf:su* mutants may function, in part, as suppressors via interactions with scaffolding protein *in vivo*.

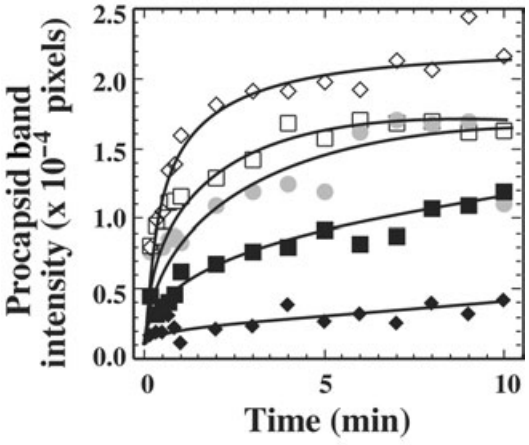

Fig. 3. *In vivo* procapsid formation with WT coat protein, *tsf* and *tsf:su* mutants. Pulse-chase experiments were performed on cells infected with phage containing WT, *tsf* and *tsf:su* coat protein. Aliquots were taken at times from 10 s after chase to 10 min after chase. Samples were then run on 1.2% agarose gels to separate procapsids from cellular proteins. Procapsid bands were quantified using densitometry. The raw data for the amount of radiolabelled proteins incorporated into procapsids as a function of time is shown. This plot represents a single experiment run on 1 day and on the same gel in order to accurately compare assembly from the phage strains. Symbols represent data for WT (closed grey circles), S223F (closed black squares), S223F:T166I (open squares), F353L (closed black diamonds) and F353L:T166I (open diamonds) phage. The lines are drawn to aid the eye, and are not meant to represent the fit of the data to any model. These data are a representative sample. This particular set was chosen because permissive conditions reflect minimal aggregation and maximal assembly of coat protein. This experiment was repeated using different strains of bacteria, slightly different temperatures or different phage strains. In all cases, the overall trend was the same.

Tsf:su *coat proteins utilize scaffolding protein more effectively during* in vitro *assembly than their* tsf *parents*

An advantage of *in vitro* assembly reactions is that production of procapsids is solely dependent on the purified proteins added to the reaction mixture. *In vitro* assembly of procapsids simply requires the addition of scaffolding protein to coat protein. In previous experiments, *tsf:su* coat proteins were shown to be more assembly competent *in vitro* as compared with their *tsf* parents using light scattering to detect procapsid assembly (Aramli and Teschke, 2001). These experiments were performed with scaffolding protein concentrations in excess over coat protein. Here, we wanted to characterize the interactions between coat protein and scaffolding protein by determining the limiting concentration of scaffolding protein for assembly. We reasoned that if the assembly of a mutant

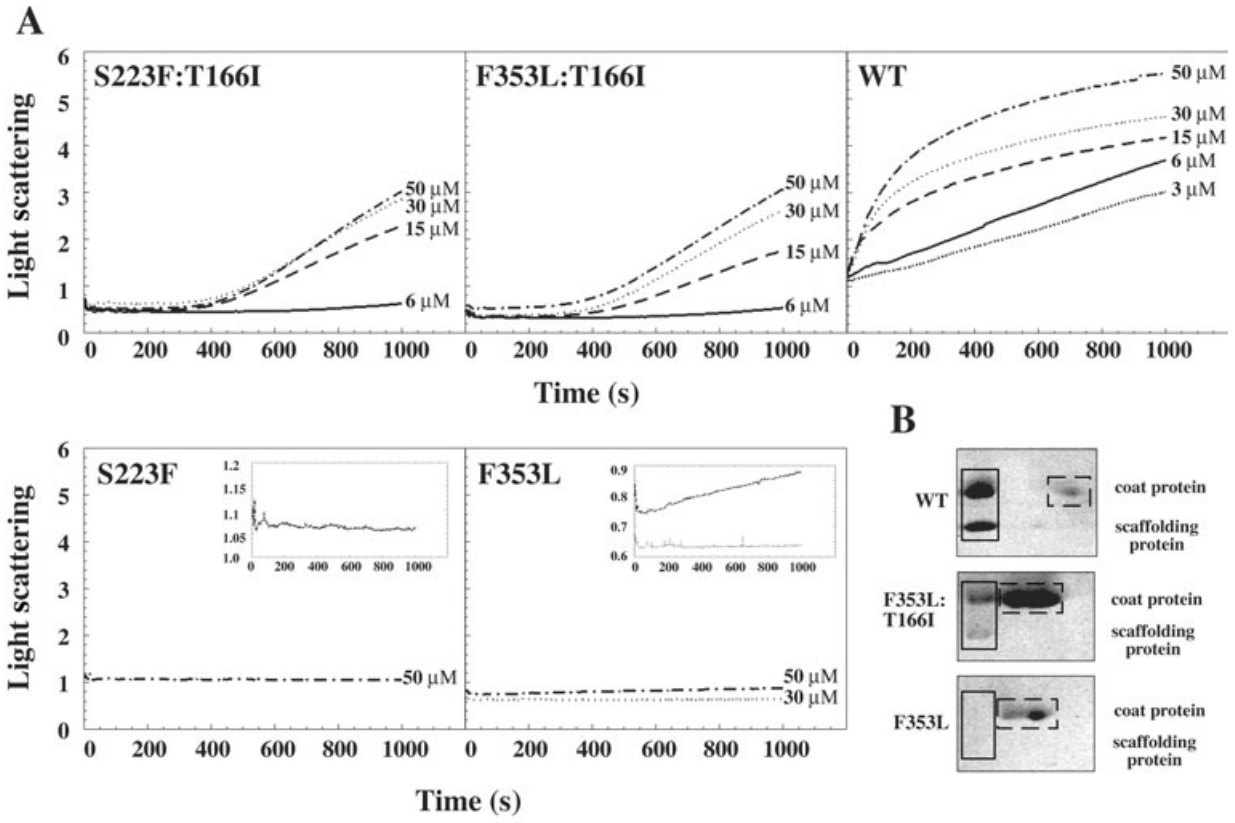

Fig. 4. *In vitro* interaction of scaffolding protein with WT, *tsf* or *tsf:su* coat proteins.
A. Light scattering data for assembly experiments using WT, *tsf* and *tsf:su* coat proteins. Coat protein monomers were mixed with varied concentrations of purified scaffolding protein as described in *Experimental procedures*.
B. A portion of a silver-stained second dimension gel from the cross-linking experiments. The solid box shows the area of scaffolding protein:coat protein cross-linking, and the dotted box indicates the region of coat protein:coat protein cross-linking. These cross-linking samples are representative data from many sets. The second dimension gels for S223F and S223F:T166I were very similar (data not shown).

coat protein were more dependent on scaffolding protein, then assembly of that mutant would require more scaffolding protein than WT coat protein. We were also interested in using the formation of the assembly nucleus as a sensitive measure of the ability of coat protein and scaffolding protein to interact. In assembly experiments, we are able to observe the time for nucleation of procapsid assembly for the different coat proteins by monitoring the lag time for the assembly reaction. The nucleation time is a combination of both the time required to form an assembly nucleus and to assemble a product large enough to be detected.

Wild type, *tsf* or *tsf:su* coat proteins were held at a fixed protein concentration of ≈12 μM monomer, and assembly was monitored by light scattering as a function of time with scaffolding protein concentrations ranging from ≈3 μM to ≈50 μM at 20°C. The temperature was set to 20°C as this is the standard temperature for *in vitro* assembly reactions (Prevelige *et al.*, 1988) (Fig. 4). The coat protein concentration was set lower than our previously published experiments, which were performed at 18 μM, to increase the nucleation time (Aramli and Teschke, 2001). WT coat protein readily formed procapsids *in vitro* without demonstrating any lag in assembly, even at 3 μM scaffolding protein. The dead time for the manual mixing experiment described here is ≈5–7 s; therefore, the nucleation time required for procapsids formed from WT coat protein was less than 7 s. For procapsids formed from either of the *tsf:su* coat proteins, the lag before assembly begins was ≈400 s, considerably longer than for procapsids formed from WT coat protein. This experiment shows that the *tsf:su* coat proteins were able to interact with scaffolding protein both during formation of the nucleus and in propagation of assembly, although higher concentrations of scaffolding protein were required than for assembly of WT coat protein. Conversely, both the rate and overall yield of procapsids formed were much lower for both *tsf* coat proteins. A lag time was not observed for the *tsf* coat proteins because so little assembly occurred. As controls, samples of assembly reactions performed with 50 μM scaffolding protein were run on agarose gels to confirm that the increase in light scattering resulted from assembly of pro-

capsids and not from aggregation (data not shown). In addition, no changes in light scattering were observed when coat protein or scaffolding protein was incubated alone. Thus, we can conclude that the suppressor substitution, T166I, allows for improved interactions with scaffolding protein, though the *tsf:su* proteins do not utilize scaffolding protein as effectively as WT coat protein.

To assess whether or not the changes in assembly competence for the different coat protein mutants directly correlated with changes in interactions with scaffolding protein, cross-linking experiments were performed. The cross-linking experiments were performed with purified coat protein monomers and scaffolding protein at 0.2 mg ml^{-1} each (4.3 and 6.1 μM respectively). This concentration of coat protein is below the critical concentration for assembly (Teschke and Fong, 1996). In addition, light scattering experiments were performed and showed that assembly of procapsids does not occur under these conditions for WT or any of the mutant coat proteins (data not shown). DTSSP is an amine reactive cross-linker that has a disulphide bond that can easily be reduced. The cross-linked samples were run on SDS gels without reducing agent to separate the high-molecular-weight cross-linked species from monomeric proteins. Entire lanes containing cross-linked species were excised and soaked in sample buffer containing β-mercaptoethanol and applied horizontally to a second SDS gel. Therefore, the cross-linked species are released from each other and the proteins are separated based on their individual molecular weights. In this manner, we are able to determine whether cross-links between coat protein and scaffolding protein were evident. In addition, we were able to visualize coat protein:coat protein interactions.

Pictures of the second dimension SDS gels are shown in Fig. 4B. Significant amounts of cross-linking of coat protein to scaffolding protein were detected for WT and F353L:T166I. In contrast, little cross-linking of coat protein to scaffolding protein was observed for the *tsf* mutant, F353L. However, all samples demonstrated coat protein:coat protein interactions, which indicates that these coat proteins are able to interact with each other. Cross-linking experiments for S223F and S223F:T166I coat proteins were performed and showed similar results (data not shown). From these *in vitro* experiments, we concluded that scaffolding protein had enhanced physical interaction with the *tsf:su* coat proteins as compared with their *tsf* parents, indicating that the *su* substitution may, in part, function by enhancing interactions with scaffolding protein.

Tsf:su *mutant coat proteins induce GroEL and GroES expression* in vivo

Our burst experiments and experiments by Aramli and Teschke (1999) indicate that GroEL and GroES are required for phage production by the *tsf:su* coat proteins (Aramli and Teschke, 1999); however, additional GroEL and GroES do not enhance phage production by the *tsf:su* coat proteins (Fig. 1A and B). Additionally, from *in vitro* experiments, we know that the folding of the *tsf:su* proteins remains defective (Doyle *et al*., 2004). Taken together, these observations suggest that the *tsf:su* coat proteins use the cellular amounts of GroEL and GroES so efficiently that additional amounts no longer help in the folding of these coat proteins. Here we investigated whether the *tsf:su* proteins induce chromosomal GroEL and GroES expression so that the extra plasmid-encoded GroEL and GroES no longer aid in efficient folding.

Pulse-chase experiments were used to radiolabel infected cells at 30°C as described above. The radiolabelled cell lysates were separated by sucrose gradient sedimentation and then analysed by SDS-PAGE. The amount of radiolabel incorporated into both coat protein and GroEL bands was quantified by densitometry. We determined the percentage of radiolabelled coat protein that was present as monomers, GroEL bound and assembled structures.

Autoradiographs from the SDS gels of sucrose gradient fractions where GroEL sediments are shown in Fig. 5A. We observed that the level of GroEL expression varied by the type of coat protein present in the infection. Samples containing WT coat protein showed the lowest level of GroEL expression, while both *tsf* mutants showed increased expression, which was also observed by Gordon *et al*. (1994). Moreover, both *tsf:su* mutants showed higher GroEL expression levels than their respective parents. In order to more accurately quantify our observations, the ratio of radiolabelled GroEL to radiolabelled soluble coat protein was determined (Fig. 5B). In different experiments, the radioactive decay and the number of cells infected may vary. Instead of plotting the raw intensity for each protein, the ratio of GroEL to coat protein intensities was used, which normalizes the different experiments. This enables us to directly and accurately compare samples. WT coat protein, which has no requirement for GroEL (Nakonechny and Teschke, 1998), showed the smallest GroEL induction. Therefore, we set the ratio of GroEL:WT coat protein to be equal to one and all other ratios were normalized to WT values. If the coat protein of one mutant induces GroEL expression more than another mutant, we would see a higher ratio (more GroEL molecules present per one coat protein). As expected, the *tsf* coat proteins induced higher GroEL levels as compared with WT. Unexpectedly, both *tsf:su* mutants exhibited even higher ratios of GroEL per coat protein, and therefore induced more GroEL than their respective *tsf* parents (Fig. 5B). This experiment was repeated multiple times at 30°C, using various phage strains and bacterial

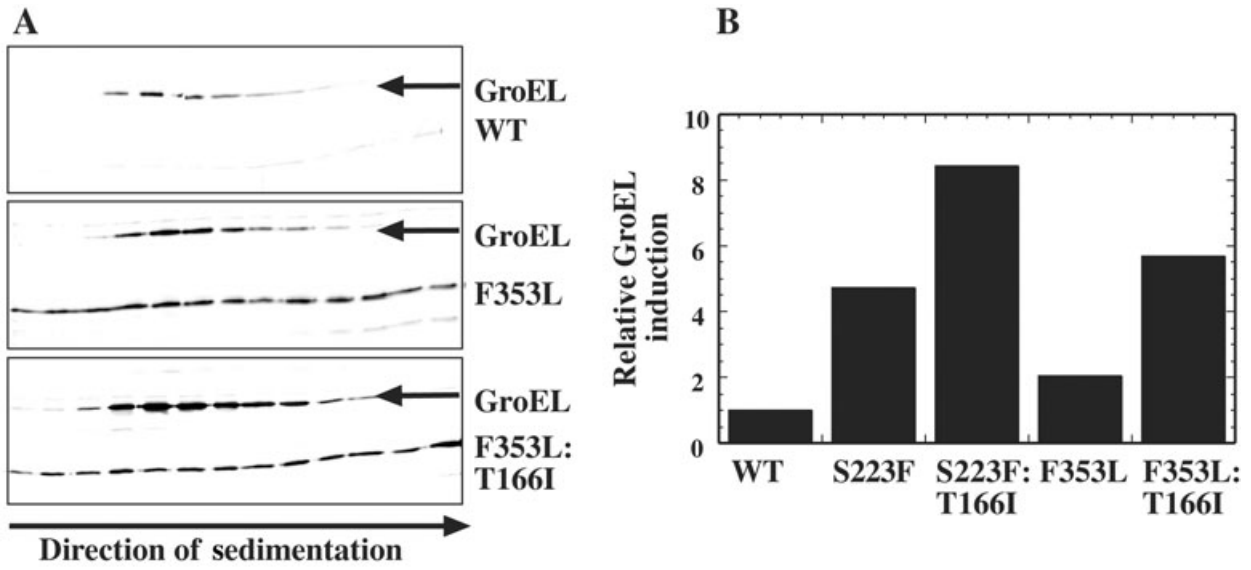

Fig. 5. Sucrose gradient sedimentation profiles of WT, *tsf* and *tsf:su* coat protein bound to GroEL. Pulse-chase experiments were performed on cells infected with phage that carried *tsf*, *tsf:su* or WT coat protein as described in *Experimental procedures*. Linear 5–20% sucrose gradients were run, fractionated and the samples were run on 10% SDS-acrylamide gels.
A. The autoradiography illustrates the middle of the gradient, spanning lanes 10–22, where GroEL normally sediments. The gradients shown are representative data from many experiments. Protein bands from the sucrose gradient samples were quantified using densitometry. The total amount of labelled soluble coat protein and labelled GroEL for each mutant was calculated. The ratio of labelled GroEL per labelled soluble coat protein for each mutant was normalized to the WT data.
B. The relative GroEL concentration under normal cellular conditions. These experiments were repeated multiple times under different conditions (varied bacterial strains and varied temperatures), with all experiments demonstrating the same overall trend.

strains. In addition, this experiment was performed at a non-permissive temperature of 36°C (data not shown). In all cases, the same trend was observed. We conclude that *tsf:su* coat proteins induce more GroEL expression relative to their *tsf* parents.

As GroEL is induced by infection with phage P22, it is likely that the other heat shock proteins such as DnaK and DnaJ that also use the σ^{32} heat shock promoter are also induced, as has been observed in cells infected with phage lambda (Drahos and Hendrix, 1982). When phage P22 carrying WT, *tsf* or *tsf:su* coat proteins were plated on cells that overexpress DnaK and DnaJ, no changes in phage production were observed compared with normal cellular conditions (data not shown). So, while we cannot absolutely exclude the possibility that these other chaperones play a role in the folding and assembly of the *tsf:su* coat proteins, it is unlikely to be the case.

Tsf:su *mutant coat proteins recruit GroEL* in vivo

As GroEL expression levels are increased by the presence of *tsf:su* coat proteins, it is reasonable that the chaperone is being induced in response to the production of aggregation-prone coat protein intermediates, even at permissive temperatures. Indeed, the *tsf* mutants bind GroEL with higher affinity than WT coat protein, as demonstrated from *in vitro* binding assays (de Beus *et al.*, 2000), and are released more slowly from GroEL *in vivo* (Nakonechny and Teschke, 1998). Here we performed an *in vivo* experiment to test whether the *tsf:su* mutant coat proteins not only recruit GroEL by inducing expression but also have an increased requirement for GroEL compared with their *tsf* parents.

In order to assess whether or not the *tsf* and *tsf:su* mutant coat proteins bind GroEL differently, the per cent of soluble coat protein bound to GroEL was determined for each variant (Fig. 6) by performing pulse-chase experiments followed by sucrose gradient sedimentation, as described above. These experiments were performed at 30°C to minimize aggregation. We observed very little soluble WT coat protein binding to GroEL, which is consistent with both *in vivo* and *in vitro* experiments that have shown that phage carrying WT coat protein do not require GroEL for proper folding and assembly (Nakonechny and Teschke, 1998; Doyle *et al.*, 2003). *In vivo* the *tsf* mutants can be rescued at elevated temperatures by interaction with GroEL and GroES (Gordon *et al.*, 1994; Nakonechny and Teschke, 1998; Aramli and Teschke, 1999), and therefore we expected that these mutants would show increased binding by the chaperone as compared with WT coat protein. As shown in Fig. 6, a higher percentage of the *tsf* coat proteins did bind to GroEL, therefore our *in vivo* results are consistent with previous experiments. The *tsf:su* mutants showed even more binding than their *tsf*

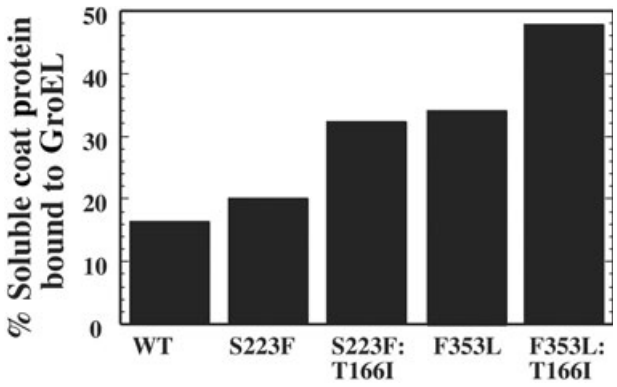

Fig. 6. Binding of WT, *tsf* and *tsf:su* coat proteins by GroEL. Phage-infected cells were pulse-chased and sucrose gradients were performed on the lysates as described in *Experimental procedures*. The amount of coat protein for each mutant in each fraction was calculated and the per cent of the soluble coat protein present in the fractions containing GroEL is shown. These experiments were repeated multiple times under different conditions (varied bacterial strains and varied temperatures), with all experiments demonstrating the same overall trend.

parents, which again suggests improved interactions with GroEL and GroES. Although these experiments cannot distinguish whether the differences observed result from changes in affinity or release rates, clearly changes in interactions with GroEL and GroES are one part of how the *su* substitution improves the folding yield of the *tsf* coat proteins.

In total, we conclude that the second-site suppressor substitution, T166I, functions to alleviate the *tsf* phenotype of the *tsf* coat proteins by improved kinetic partitioning between aggregation and the native state via interactions with a chaperone network that consists of GroEL, GroES and P22 scaffolding protein.

Discussion

Previous *in vitro* studies from our laboratory showed that P22 *tsf:su* coat proteins were more aggregation-prone than their *tsf* parents (Aramli and Teschke, 2001). Through *in vitro* folding and unfolding experiments, we have shown that the *tsf*:*su* proteins have a more populated folding intermediate because of changes in folding and unfolding rates, which explains why the *tsf*:*su* proteins are more aggregation-prone than their *tsf* coat protein parents (Doyle *et al*., 2003; 2004). These results are surprising because the typical effect of a suppressor substitution is to decrease aggregation (Mitraki *et al*., 1991; Tsai *et al*., 1991Schuler and Seckler, 1998; Sideraki *et al*., 2001).

If the second-site suppressors do not function by alleviating aggregation, how do they modify the *tsf* phenotype for coat protein? Additional *in vitro* experiments led us to propose that the mechanism of suppression might result from the interaction between coat protein and scaffolding protein (Aramli and Teschke, 2001). In the presence of excess scaffolding protein, the *tsf:su* coat proteins exhibited enhanced procapsid production as compared with their *tsf* parents *in vitro* (Aramli and Teschke, 2001). Here we confirmed that the *su* substitution, T166I, functions in part via enhanced scaffolding protein interactions *in vivo*. The *su* substitution also acts through recruiting the molecular chaperone complex, GroEL and GroES. The correlation between our previous *in vitro* results and the *in vivo* data presented here provides a strong argument for our proposed model of kinetic partitioning between aggregation of folding intermediates and trapping of the native state as a means of alleviating a folding defect for P22 coat protein *in vivo*.

Chaperones in protein folding and in capsid assembly

GroEL and GroES are important in the folding and assembly of P22 coat protein variants. Here, and in previous experiments, we have shown that the *tsf* and *tsf*:*su* coat proteins require GroEL and GroES for proper folding, but WT coat protein folds independently of these chaperones. This conversion from non-substrate to a substrate of GroEL probably results from an increase in the population of intracellular hydrophobic folding intermediates (Doyle *et al*., 2003; 2004).

Although many researchers have attempted to understand what drives the interaction of a substrate polypeptide with GroEL, no consensus has arisen from these studies. Strength of substrate polypeptide interaction with GroEL could result from either differences in conformation or differences in the kinetics of folding. Both hydrophobic and ionic interactions have been implicated in the binding of substrate polypeptides to GroEL, although hydrophobic interactions are the more likely requirement for substrate recognition as indicated by structural studies (Schmidt and Buchner, 1992; Zahn *et al*., 1994; Perrett *et al*., 1997; Persson *et al*., 1999). GroEL has been observed to bind to molten globule intermediates, as well as late folding intermediates (Martin *et al*., 1991; Hayer-Hartl *et al*., 1994; Katsumata *et al*., 1996; Goldberg *et al*., 1997; de Beus *et al*., 2000). Other studies comparing the interaction of homologous proteins with GroEL have implicated slow folding kinetics as crucial to substrate polypeptide recognition (Tieman *et al*., 2001). Frieden and co-workers have suggested that it is the rate of association and dissociation of the substrate polypeptide with GroEL relative to its refolding rate that controls GroEL-assisted folding (Frieden and Clark, 1997; Clark and Frieden, 1999). Our *in vivo* and *in vitro* data are not consistent with the notion that slow folding kinetics are the cause for interaction with GroEL. The *tsf* and *tsf*:*su* coat proteins actually fold to the native state more quickly than WT coat protein; however, they also unfold from the native state to intermediates

greater than 2000-fold faster than WT coat protein. However, Frieden and co-workers also suggest that GroEL binding might be caused by inefficient folding because of aggregation (Clark and Frieden, 1999), which is consistent with our data. Based on our experiments, we postulate that it is actually the increase in the population of intermediates *in vivo* that controls GroEL/ES-assisted folding of substrate polypeptides.

Chaperones have a widespread role in the folding of many proteins of viruses and bacteriophage. For example, the GroEL and GroES complex rescues the folding of diverse proteins including the gene E product, which is the capsid protein in lambda phage (Sternberg, 1973a,b; 1976), the major capsid protein (gene product 23) for T4 (Zeilstra-Ryalls *et al*., 1991) and the tail assembly for T5 (Tilly *et al*., 1981). In eukaryotes, molecular chaperones such as hsc70 have a widespread role in the life cycle of viruses such as polyomavirus (Chromy *et al*., 2003), adenovirus (Saphire *et al*., 2000) and rotavirus (Zarate *et al*., 2003). Hsc70 aids in capsid localization for both adenovirus and polyomavirus. For adenovirus, hsc70 mediates docking of the major capsid protein to the nuclear pore, thus facilitating DNA packaging, which is necessary for infection (Saphire *et al*., 2000). In polyomavirus, localization of VP1 (viral coat protein) to the nucleus is mediated by hsc70 (Chromy *et al*., 2003). Hsc70 is believed to function by aiding cell entry of the cleaved viral protein, VP5, a process that is necessary for rotavirus biogenesis (Zarate *et al*., 2003).

Scaffolding proteins in virus morphogenesis

Scaffolding proteins are required for proper assembly of many viruses, including herpesvirus and adenovirus, as well as other dsDNA bacteriophage (Morin and Boulanger, 1984; Casjens and Hendrix, 1988; King and Chiu, 1997). For these viruses and phage, scaffolding proteins help direct the proper assembly of capsids, but they are not required for infectivity as they are removed during maturation (Dokland, 1999). Scaffolding proteins appear to generally have two functions in capsid assembly: assisting in formation of the assembly nucleus, and organizing the structure and completion of the capsid (Fane and Prevelige, 2003). In a sense, scaffolding proteins can be thought of as chaperones as they inhibit inappropriate interactions of coat proteins during assembly that lead to aberrant capsid structures (Dokland, 1999). Here, we show that P22 scaffolding protein may also play a role in folding by trapping the native state of unstable monomeric coat proteins in the capsid.

Other suppressor substitutions function via different mechanisms from the su *substitution in P22 coat protein*

Suppressor substitutions exist that function to decrease aggregation caused by a single amino acid defect for various proteins such as human LAR, a transmembrane receptor-like protein, chloramphenicol acetyltransferase, β-lactamase and P22 tailspike protein (Mitraki *et al*., 1991; Tsai *et al*., 1991; Schuler and Seckler, 1998; Sideraki *et al*., 2001). The mode of action for the suppressors is varied for these four proteins. In human LAR, the suppressor decreases aggregation through an increase in protein production (Tsai *et al*., 1991). A suppressor for chloramphenicol acetyltransferase functions by improving thermodynamic stability of the native protein (Van der Schueren *et al*., 1998). A P22 tailspike protein suppressor works by alleviating steric hindrance in the native conformation (Beißinger *et al*., 1995). Finally, a β-lactamase suppressor functions by increasing solubility of the molecule (Sideraki *et al*., 2001).

The suppressors described above rely solely on improvements made within the defective proteins themselves. Therefore, interactions with other proteins are irrelevant in terms of alleviating defects in their folding pathways. P22 coat protein suppressors act in a novel way. Unlike the other second-site suppressors, the *su* substitutions in P22 coat protein do not primarily work by improving monomeric coat protein stability, thereby leading to a decrease in aggregation at non-permissive temperatures (Aramli and Teschke, 2001; Doyle *et al*., 2003). Instead, the *tsf* phenotype caused by single amino acid substitutions in coat protein is alleviated by the suppressor substitution because of a shift in the kinetic partitioning between aggregation and procapsid assembly through direct involvement with molecular chaperones. Productive folding is favoured through interactions with both the folding chaperones, GroEL and GroES, and the assembly chaperone, scaffolding protein, which drives coat protein towards procapsid formation. The combination of these effects culminates in a novel kinetic mechanism.

Our model for the folding and assembly of WT, tsf*, and* tsf:su *coat proteins* in vivo

Based on the data presented here and data from other experiments, we propose the following model (Fig. 7). WT, *tsf* and *tsf:su* coat proteins have been shown to fold to their native conformation via folding intermediates (Teschke and King, 1993; 1995; Aramli and Teschke, 2001). It is a late folding intermediate that is believed to interact with GroEL (de Beus *et al*., 2000). WT coat protein does not require GroEL and GroES for folding (Nakonechny and Teschke, 1998). WT coat protein folds rapidly to the native state and has a very slow unfolding rate from the native state to the intermediate, N⇒I (Anderson and Teschke, 2003). Therefore, the population of the intermediate is small, and it does not aggregate significantly, suggesting the reason why WT coat protein

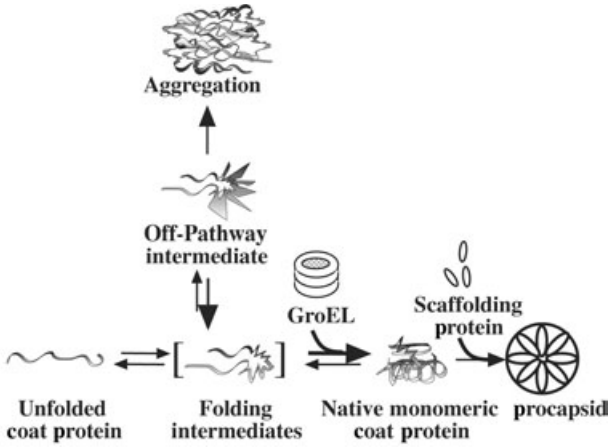

Fig. 7. Kinetic partitioning of P22 coat protein through a chaperone network. WT coat protein folds to the native state via an intermediate. *Tsf* and *tsf:su* mutant coat proteins fold to an altered, yet assembly competent native state, also via an intermediate. This intermediate state can form non-productive aggregates. WT coat protein intermediates do not significantly aggregate, and do not require GroEL and GroES, but the presence of the chaperone reduces aggregation of intermediates for both the *tsf* and *tsf:su* coat proteins. The native state of coat protein can interact with scaffolding protein to produce a precursor structure known as the procapsid. Darker arrows represent the flow of through the reaction.

does not require GroEL and GroES. Instead, WT coat protein efficiently populates the native state, where it can effectively interact with scaffolding protein to produce procapsids.

Unlike WT coat protein, the *tsf* coat proteins rapidly flicker between native and intermediate structures, populating the intermediate state (Doyle *et al*., 2003). The intermediate also rapidly unfolds. Therefore, the *tsf* coat protein intermediates are likely to aggregate, which explains why the *tsf* coat proteins require GroEL and GroES for folding. Less native *tsf* coat protein is available to interact with scaffolding protein to produce procapsids because of the rapid folding and unfolding. The *tsf:su* mutants have a slower unfolding rate of the native and intermediate states, increasing the population of both (Doyle *et al*., 2004). The folding to the intermediate states is faster, thereby increasing their concentration, which is why they are more aggregation-prone than their *tsf* coat protein parents. GroEL interactions with the *tsf:su* mutants aid in alleviating aggregation by driving the reaction towards proper folding. Once in the native conformation, the *tsf:su* coat proteins unfold slowly compared with their *tsf* parents. These two combined factors of slower unfolding and increased molecular chaperone interactions allow for a greater pool of native coat protein. As more native coat protein is available, interactions with the assembly chaperone, scaffolding protein, traps the native state in procapsids, pulling the equilibrium towards the direction of proper folding and assembly. Thus, we propose that the suppressor substitution functions to alleviate aggregation by a novel mechanism that consists of a network of chaperones, where both the scaffolding protein of bacteriophage P22 and GroEL and GroES act in a concerted manner to fold and assemble defective coat proteins.

Experimental procedures

Bacteria

Salmonella typhimurium strain DB7136 (leuA414^{-}am, hisC525^{-}am, su^{-}) and the su^{+} derivative DB7155 (supE20^{-} gln, leuA414^{-}am, hisC525^{-}am) have been previously described by Winston *et al*. (1979). Strains either carried the pOF39 plasmid, which carries the *groEL/S* operon behind its own promoter (Fayet *et al*., 1986) and is ampicillin resistant at 100 μg ml^{-1}, or were studied without the plasmid as a control.

Bacteriophage

The P22 bacteriophage used in these studies were either WT in gene 5, which codes for coat protein, or carried mutations which result in the *tsf* amino acid substitutions serine at position 223 to phenylalanine (S223F), or phenylalanine at position 353 to leucine (F353L). In addition, phage with the global suppressor substitution (*su*), threonine for isoleucine at position 166 (T166I), were used in conjunction with the F353L and S223F mutations (*tsf:su*). For various experiments, the phage might carry additional amber mutations in genes 3, 8 and 13 to prevent DNA packaging, scaffolding protein production or lysis respectively. Phage without amber mutations were used as controls. All P22 strains carried the c1-7 allele to prevent lysogeny.

WT, *tsf* and *tsf:su* phage containing an amber mutation in gene 8 were used to study the fate of coat protein when levels of scaffolding protein were decreased. Phage containing an amber mutation in gene 8 were plated on *Salmonella* DB7155 carrying the *supE* gene so that the amber mutation was suppressed by the nonsense tRNA suppressor. Using pulse-chase experiments using radioactive amino acids, we determined that 25% of normal levels of scaffolding protein were produced when the 8^{-}am phage were grown on DB7155.

Media

Luria broth (LB, from Invitrogen Life Technologies) was used to support bacterial growth for plating experiments, burst experiments, and for the preparation of phage stocks. For pulse-chase experiments, minimal media was used. Minimal media contains M9 plus 1 mM $MgSO_4$, 1 μM $FeCl_3$ and $CaCl_2$, 1.2% glucose, 0.008% leucine and 0.003% histidine. M9 media contains 1.28% $NaHPO_4$, 0.3% KH_2PO_4, 0.05% NaCl and 0.1% NH_4Cl. For storing phage stocks, M9 with 2 mM $MgSO_4$ was used.

Phage burst size determination

Phage either were WT in gene 5 or carried *tsf* or *tsf:su*

mutations as described previously. The phage in some cases also carried an amber mutation in gene 8 to decrease scaffolding protein levels. DB7155 (carrying *supE*) cells with and without the pOF39 plasmid were grown in LB at temperatures from 30°C to 39°C until the density was 2×10^8 cells per ml. Phage infection was performed at a multiplicity of infection (moi) of 10 for all phage strains. After 2 h of infection, an aliquot of infected cells was taken and diluted with M9 media that contained saturating amounts of $CHCl_3$ to lyse the cells, and were plated to determine the number of phage produced per cell at each temperature studied. The number of phage produced per cell was then corrected for the number of cells infected. Only infections that resulted in less than 7% of cell survival were used for burst experiments. This ensures that burst size determination was not altered by a second round of infection of cells that were not originally infected at the start of the experiment. Error bars represent the standard deviation from three data sets (Fig. 1).

To confirm that our observations in the burst experiments did not simply result from the presence of any amber mutation carried by the phage, the burst size for an infection with a phage containing an amber mutation in gene 3, which codes for one of the DNA packaging ATPases, was determined. These infections produced similar numbers of phage per cell as phage without any amber mutations when grown on *Salmonella* with the *supE* amber suppressor. Controls were also performed with a *Salmonella* strain carrying a different nonsense tRNA suppressor, *supF*, again with both normal and overexpressed levels of GroEL/ES. Results from this strain were consistent with *Salmonella* carrying the *supE* gene. From the controls, we conclude that the changes in phage production do not result from the type of amber suppression.

In vitro *procapsid assembly reactions*

Assembly of procapsids was performed as described previously (Aramli and Teschke, 2001) with the following modifications: the final coat protein concentration was 0.55 mg ml^{-1} (≈12 μM) and the final scaffolding protein concentrations ranged between 1.7 mg ml^{-1} and 0.1 mg ml^{-1} (50 μM to 3 μM). An Amino-Bowman 2 spectrofluorometer was used to monitor the light scattering by setting the excitation and emission wavelengths to 350 nm with both bandpasses set to 4 nm. The temperature was set to 20°C using a thermostatted water bath.

Cross-linking experiments

Purified coat protein monomers and scaffolding protein monomers were obtained as described previously (Fuller and King, 1980; Teschke and Fong, 1996). Coat protein monomers and scaffolding protein were mixed together in 20 mM sodium phosphate, pH 7.6, at a 1:1 volume ratio to yield final concentrations of 4.3 μM and 6.1 μM respectively. The reaction was performed at 20°C. A 25 molar excess of DTSSP [3,3′ Dithio bis(sulphosuccinimidylpropionate), Pierce Biotechnology] over the total protein concentration was added to the mixture to initiate the cross-linking reaction. Samples were taken at 5 min and quenched by the addition of SDS sample buffer, which contained 200 mM Tris, pH 6.8, 210 mM SDS, 30% glycerol. SDS-PAGE (7.5% acrylamide) was used to separate the cross-linked complexes. The lanes were cut from the gel, and soaked with sample buffer (20 mM Tris, 3 mM SDS, 20% glycerol, 0.7 M β-mercaptoethanol, pH 7.8) to disrupt the disulphide bonds. The excised lanes were applied horizontally to a 10% SDS-PAGE (Josefsson and Randall, 1983). The gels were silver-stained and developed for the same amount of time (Rabilloud *et al*., 1988).

Pulse-chase experiments to monitor procapsid assembly in vivo

Overnight cultures were grown in minimal media from a single isolated colony of DB7136. The overnight cultures were used to inoculate a culture in minimal media, grown at the experimental temperature [either 30°C (permissive) or 36°C (non-permissive)]. The cells were grown to a density of 2×10^8 cells per ml, and then infected with an moi of 17, which produces maximal infection of the cells. The infected cultures were grown for 43 min, followed by a 1 min pulse of 20 μCi ml^{-1} [^{35}S]-methionine and [^{35}S]-cysteine protein labelling mix (10 mCi ml^{-1}, NEN Life Science Products). After 1 min, a chase of unlabelled cysteine/methionine at a final concentration of 450 μM each was added.

To follow procapsid synthesis, samples were taken with time starting 10 s after chase. The aliquots were transferred immediately into a microfuge tube on ice, and then frozen at –20°C. The radiolabelled samples were thawed on ice and incubated for 1 h with 0.1% Triton, 5 mM EDTA, 20 mM $MgSO_4$ and 100 μg ml^{-1} DNase. Samples were then frozen and thawed twice to be sure of complete lysis. Procapsid synthesis was monitored by running the cell lysates on a 1.2% agarose gel (Serwer and Pichler, 1978; Serwer *et al*., 1986; Nakonechny and Teschke, 1998). To determine total amount of label incorporated into GroEL and coat protein, an aliquot of the cell lysates were run on 10% SDS gels. The amount of aggregated radiolabelled coat protein was determined by performing pellet/supernatant separations according to Gordon and King (1993). The quantification of protein bands and procapsids was performed by densitometry of the autoradiographs using a Kodak EDAS system.

Sucrose gradient centrifugation

Labelled cell lysates for WT and all mutants were prepared according to the protocol above, except that aliquots were taken 30 s after the chase. After the freeze–thaw lysis, the lysates were spun for 30 s in a microcentrifuge to pellet any unlysed cells. The supernatant, 200 μl, was applied to a 2.2 ml 5–20% sucrose gradient and run in a RP55S rotor in a Sorvall RC M120EX centrifuge for 3 h and 10 min at 50 000 r.p.m. at 4°C. The linear sucrose gradients were made using a Gradient Master Model 106 (Biocomp Instruments). The gradients were hand-fractionated from the top taking 100 μl aliquots. The samples were run on a 10% SDS-polyacrylamide gel. Quantification of the autoradiography was performed as described above to determine the amount of soluble coat protein bound to GroEL, as well as the amount

of scaffolding protein per coat protein in the procapsids. The intensity of the coat protein and scaffolding protein in the procapsids was corrected for the number of labelled amino acids: 13 methionine and one cysteine residues for coat protein, and eight methionine residues for scaffolding protein (Eppler *et al*., 1991). For GroEL induction, the total radiolabelled GroEL expressed for each mutant was calculated. The ratio of GroEL to total soluble coat protein for the same mutant was calculated. 'Soluble' coat protein is considered to be any coat protein that is not in the last fraction of the sucrose gradient, which contains procapsids and aggregated structures. Therefore, soluble coat protein consists of free monomeric coat protein, as well as GroEL-bound coat protein. In order to correct for small differences between samples (such as radioactive decay, infection levels, time of exposure), this ratio was used to normalize the data. All ratios were normalized to WT.

Acknowledgements

We would like to thank Dr Jonathan King for helpful discussions regarding this project. In addition, we are grateful to Dr Shannon Doyle for assistance with some of the experiments, and the critical reading of this manuscript. Finally, we would thank Eric Anderson for the critical reading of this manuscript. This project was sponsored by NIH Grant GM53567 to C.M.T.

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