GroEL and GroES Control of Substrate Flux in the \textit{in Vivo} Folding Pathway of Phage P22 Coat Protein* \hfill
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Our present understanding of the action of the chaperonins GroEL/S on protein folding is based primarily on \textit{in vitro} studies, whereas the folding of proteins in the cellular milieu has not been as thoroughly investigated. We have developed a means of examining \textit{in vivo} protein folding and assembly that utilizes the coat protein of bacteriophage P22, a naturally occurring substrate of GroEL/S. Here we show that amino acid substitutions in coat protein that cause a temperature-sensitive-folding (\textit{tsf}) phenotype slowed assembly rates upon increasing the temperature of cell growth. Raising cellular concentrations of GroEL/S increased the rate of assembly of the \textit{tsf} mutant coat proteins to nearly that of wild-type (WT) coat protein by protecting a thermolabile folding intermediate from aggregation, thereby increasing the concentration of assembly-competent coat protein. The rate of release of the \textit{tsf} coat proteins from the GroEL/S-coat protein ternary complex was approximately 2-fold slower at non-permissive temperatures when compared with the release of WT coat protein. However, the rate of release of WT or \textit{tsf} coat proteins at each temperature remained constant regardless of GroEL/S levels. Thus, raising the cellular concentration of GroEL/S increased the amount of assembly-competent \textit{tsf} coat proteins not by altering the rates of folding but by increasing the probability of GroEL/S-coat protein complex formation.

Characterizing the folding of polypeptides \textit{in vivo} is difficult because of the transient nature of their folding intermediates and because the intracellular environment can influence the kinetic partitioning between productive and non-productive folding pathways (1). Consequently, most studies of the mechanism of protein folding have been conducted \textit{in vitro} using defined assay conditions. Recently, there has been much attention given to a class of proteins called molecular chaperones, which assist folding polypeptides in reaching their native structure. It is believed that the chaperonins, the hsp60 sub-class of molecular chaperones, allow folding intermediates the opportunity to avoid kinetic traps by associating with misfolding polypeptide chains thereby preventing aggregation (2, 3).

The complex of GroEL/S, the prokaryotic chaperonins (4), forms as a homo-oligomeric double toroid consisting of 14 GroEL monomers (57 kDa) and a single ring cap of seven GroES monomers (10 kDa). A current debate about the mechanism of GroEL/S concerns the extent of structure in substrate polypeptides upon release from the GroEL inner chamber (for review, see Ref. 2). Regardless of the mechanism, it is believed that the increase in the yield of native protein is correlated to its rate of folding, \textit{i.e.} a polypeptide that is folding slowly is more prone to aggregation (5, 6). In order to study this hypothesis, folding kinetics for many GroEL/S substrates have been analyzed. \textit{In vitro} rates of folding have been shown to both increase (5, 7) and decrease (8) as a result of GroEL/S action. In addition, yields of citrate synthase (9) and mitochondrial malate dehydrogenase (10) have been shown to be enhanced by GroEL/S while the rates of folding remained unaffected.

To date, there has been little information about how GroEL/S affects the folding of substrate proteins within the cell. Recently, macromolecular crowding has been shown to affect the rate of release of rhodanese from GroEL/S \textit{in vitro} (3). In addition, the rates of release of rhodanese from GroEL/S and its partitioning into soluble and aggregated states have been calculated \textit{in vivo}, and the total polypeptide flux has been examined within the cell (11). To understand how the flux of a naturally occurring substrate is controlled by GroEL/S, we utilized the coat protein of phage P22 and its \textit{tsf} mutants, which misfold at high temperatures yet fold correctly at lower temperatures (12). Previous studies of the folding of WT and \textit{tsf} coat proteins conducted \textit{in vitro} have shown that WT coat protein folds into assembly-competent monomeric subunits with high efficiency (13, 14). However, the \textit{tsf} coat protein mutants fold into dimers and trimers with altered secondary and tertiary structure (15, 16). Recent experiments demonstrated that \textit{tsf} coat proteins refolded in 20 mM phosphate buffer are assembly-competent, albeit with reduced kinetics.² Thus, we will be able to compare our \textit{in vivo} folding results to folding \textit{in vitro} where defined conditions were used.

Bacteriophage P22 is a lambdoid-like phage with a capsid composed of coat protein (gene product 5) subunits arranged in a \(T = 7\) icosahedron (17). Procapsid assembly occurs by the association of 420 molecules of coat protein with 150–200 molecules of scaffolding protein (gene product 8) along with minor proteins in a nucleation dependent reaction (Fig. 1) (18–22). Therefore, any change in the kinetics of the folding of coat protein into the assembly-competent state influences the rate of nucleus formation, which subsequently affects the rate of procapsid assembly. After the formation of the procapsid, DNA packaging occurs through a headful mechanism (20) along with concomitant release of scaffolding protein and a conformational change of the procapsid from spherical to icosahedral (23, 24). Addition of several proteins, such as the plug proteins of the portal vertex (gene products 4, 10, and 26) and tailspike protein (gene product 9), then complete the infectious particle.

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³ The abbreviations used are: \textit{tsf}, temperature-sensitive-folding; WT, wild-type; Rubisco, ribulose-bisphosphate carboxylase/oxygenase.

⁴ C. M. Teschke, unpublished results.

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In order to investigate how GroEL/S controls the flux of coat protein in the cell, we have established a means of examining the productive and non-productive folding pathways in vivo. In this study, we have used the rate of formation of the procapsid to monitor the folding of coat polypeptides into the assembly-competent conformation within the cell (25). To examine the non-productive folding pathway, we used the amount of coat protein in the pellet after a short centrifugation as a barometer of aggregation. The rate of release of coat protein from the GroEL/groES coat protein ternary complex and the effect of elevated levels of GroEL/S upon this system were also determined. At high temperatures, the release rates of tsf coat protein from the GroEL/S-coat protein ternary complex were approximately 2-fold slower compared with WT coat protein, regardless of the level of GroEL/S expression. Aggregation increased as temperatures were raised, and GroEL/S overexpression decreased the amount of aggregate. We found that the rate of assembly of the tsf polypeptides was slower at high temperatures and that GroEL/S overexpression increased these assembly rates. Thus, GroEL/S overexpression increased the concentration of assembly-competent coat protein by increasing the frequency of coat protein and GroEL/S association and not by changing the rate of folding of the tsf coat protein mutants within the GroEL/S-coat protein ternary complex. These conditions likely mimic those found in a severely stressed cell.

**EXPERIMENTAL PROCEDURES**

**Bacteria—Salmonella typhimurium** strain DB7000 (leu A144am) contained either pOF39, a plasmid that carries the groEL/S operon behind its own promoter (26), or pBR322 as the control plasmid. All cells were ampicillin-resistant due to the presence of the plasmid and were grown in Luria broth or minimal media with 100 μg/ml ampicillin. In addition, the strains DB7136 (leu A144am, his C525am, supE), a derivative of DB7136 that carries the glutamine amber suppressor and allows the growth of P22 strains carrying amber mutations, were used in some plating experiments (27). The Escherichia coli strains (4, 28) DW720 (WT groEL and groES), DW716 (groEL44), DW717 (groEL59), DW721 (groEL673), DW715 (groEL784), and DW619 (groES619) were transformed with the plasmid pPR1347 (29), which encodes for the rfb gene cluster and rfc gene so that the E. coli synthesizes the O antigen needed for P22 infection. The plasmid was maintained by growing cells in media with 50 μg/ml kanamycin.

**Bacteriophage—**The P22 bacteriophage used in this study either were WT in gene 5, which encodes coat protein, or carried the tsf mutations in gene 5, which lead to the amino acid substitutions serine at position 223 for phenylalanine (S223F) or alanine at position 108 for valine (A108V) (12). All phage used possessed the cl-7 allele to prevent lysogeny. In some experiments, the phage also carried amber mutations in gene 13 to prevent cell lysis, and gene 3 to prevent DNA packaging, in order to produce procapsids (30).

**Media—**M9 media contained 0.6% NaHPO₄, 0.3% KH₂PO₄, 0.05% NaCl, and 0.01% NH₄Cl (31). Minimal media was M9 with 1 mM MgSO₄, 1 μM FeCl₃, and CaCl₂, 4% glucose, 150 μM amino acids (except methionine and cysteine), and ampicillin (100 μg/ml). For storing phage stocks, dilution fluid containing 0.1% tryptone, 0.7% NaCl, and 2 mM MgSO₄ was used (25).

**Plating Efficiency—**Phage with either WT gene 5 or gene 5 tsf mutations were plated at various temperatures on E. coli strains as mentioned above. Plating efficiencies were calculated by dividing the titer of the phage grown at each temperature and on each strain of cells by the titer produced when the phage was grown at 22 °C on DW720 (12).

**Procapsid Assembly and Shift Down Experiments**—Minimal media inoculated with an overnight culture was grown at 39 °C and concentrated to a density of 3 × 10⁸ cell/ml in fresh media. The cells were infected with phage at a multiplicity of infection of 10. After 2 h of infection, an aliquot of cells was removed and diluted in dilution fluid saturated with chloroform to lyse infected cells. Dilutions of phage were plated on DB7136 cells to determine the number of phage produced/cell in each strain at a given temperature (32).

**Phage Burst—**DB7000 carrying the plasmids pOF39 or pBR322 were grown in minimal media at temperatures ranging from 30 °C to 39 °C and concentrated to a density of 4 × 10⁷ cell/ml in fresh media. The cells were infected with phage at a multiplicity of infection of 10. After 2 h of infection, an aliquot of cells was removed and diluted in dilution fluid saturated with chloroform to lyse infected cells. Dilutions of phage were plated on DB7136 cells to determine the number of phage produced/cell in each strain at a given temperature (32).

**Procapsid Assembly and Shift Down Experiments**—Minimal media inoculated with an overnight culture was grown at 39 °C and concentrated to a density of 2.5 × 10⁸ cells/ml, chilled on ice for at least 15 min, and stored at 4 °C overnight. The cells were diluted 1:10 with minimal media and grown to 2.5 × 10⁸ cells/ml at the experimental temperature, and then chilled on ice for 15 min before infection. Phage infection was carried out at a multiplicity of infection of 15 and incubated for 40, 45, 50, 55, 60, and 65 min at 39, 37, 35, 33, 30, and 28 °C, respectively. The cells were then pulsed with [³⁵S]methionine and [³⁵S]cysteine protein labeling mix (10 μCi/ml, NEN Life Science Products) for 1 min at a final isotope concentration of 20 μCi/ml. Incorporation was halved by the addition of unlabeled Met/Cys (final concentration 17 mM) and frozen in a dry ice/ethanol bath. Samples were taken at intervals after initial infection and quickly frozen by the same method. Temperature shift down experiments were performed as described above, except aliquots were shifted from 39 °C to 30 °C for 75 min prior to rapid freezing. The thawed samples from both assembly and temperature shift down experiments were analyzed by electrophoresis on a 1.2% HGT Seakem (American Bioanalytical) agarose gel to separate procapsids from other labeled proteins (35–35). The incorporation of labeled coat protein into procapsids was analyzed using a PhosphorImager (Bio-Rad GS-525) or by scintillation counting. Samples quantified by scintillation counting were prepared by excising bands containing radiolabeled procapsids rehydrated in 300 μl of double-distilled H₂O at 60 °C for 30 min, followed by elution of the isotope in 300 μl of Solvable (DuPont). Samples were heated to 60 °C for 3 h,
and then incubated at room temperature for 24 h after the addition of scintillation mixture. For the assembly experiments, half-times were determined using the formula $x_{\text{max}} - x/\Delta x$ to calculate the fraction of correctly folded coat protein. In this formula, $x_{\text{max}}$ refers to the maximum amount of $^{35}$S label incorporated into procapsids, $x$ is the amount of isotope incorporation at a given time point, and $\Delta x$ is the total change in isotope incorporation ($x_{\text{max}} - x$). The results were then plotted on a log scale against time, and $t_{1/2}$ values were calculated by determining the time at which half-maximal incorporation occurred.

**RESULTS**

**tsf Coat Protein Mutants Require GroEL/S for Folding and Assembly**—It has been reported that GroEL/S increase the yield of a variety of proteins such as Rubisco, citrate synthase, and rhodanese when they are refolded in vitro (9, 37–39). In vivo, the tsf mutants of the coat protein of bacteriophage P22 have displayed a similar increase in phage viability with the overproduction of GroEL/S (25, 40). Since we used a different growth medium for our experiments than Gordon et al. (25), we determined the number of properly folded coat proteins produced per cell (i.e. phage burst) at a variety of temperatures in cells with normal and overproduced levels of the chaperonins in order to assess the role of GroEL/S in the enhancement of proper folding of these tsf mutant coat proteins in our conditions (Fig. 2). The ability of WT coat protein to fold and assemble remained constant over the range of temperatures in both strains of cells, indicating that there was no significant effect on the yield of correctly folded WT coat protein by the overproduction of GroEL/S. The tsf mutant S223F consistently produced larger bursts than WT phage at permissive temperatures. In cells expressing normal levels of GroEL/S, S223F and A108V exhibited a decrease in the yield of assembly-competent coat protein at their non-permissive temperatures ($\approx 37 ^\circ C$). However, when the tsf coat proteins were synthesized in cells overproducing GroEL/S at the restrictive temperatures, the level of assembly-competent coat proteins approached those seen at permissive temperatures. The amount of assembly-competent coat protein observed for the tsf mutants in cells overproducing GroEL/S increased 60–200-fold over the amount when folded in cells with normal levels of GroEL/S (Fig. 2), suggesting that the 9-fold increase of GroEL/S levels (25) overcame the folding defect in tsf mutant coat proteins, which is consistent with Gordon et al. (25).

To further characterize the effect of GroEL/S on the folding and assembly of coat protein, we examined WT and tsf mutant phage biogenesis in cells producing WT GroEL/S or cells that carry point mutations in GroEL or GroES that result in dys-functional chaperonin action (Fig. 3) (28). WT phage grew efficiently in any GroEL or GroES mutant strain, even at high temperatures, and the tsf mutants showed the same temperature-sensitive phenotype in cells producing WT GroEL/S as observed in Salmonella. However, the tsf coat protein mutants G232D, P238S, and D302G, as well as other mutant phage not shown here, exhibited a dramatic decrease in the temperature where phage growth became non-permissive on each GroEL or GroES mutant strain as compared with that observed in cells producing WT GroEL/S. These data indicate that GroEL and GroES are critical for the folding of the tsf mutant coat proteins, at least at 33 °C and above, but are not an essential requirement for the folding and assembly of WT coat protein.

**Binding of tsf Coat Proteins to GroEL**—To characterize the GroEL-coat protein ternary complex, we calculated the percent of the total coat protein initially bound to GroEL at each temperature from co-immunoprecipitation experiments. From these data we observed a small increase in the percent of WT coat protein bound to GroEL with increasing temperature. Overproduction of GroEL did not significantly change the amount of WT coat protein initially bound to GroEL (Fig. 4). These data suggest that the productive folding pathway of WT

![Figure 2](image-url)
Coat protein does not generally require GroEL/S, although WT coat protein does exhibit some affinity for GroEL, particularly at higher temperatures. The percent tsf mutant coat protein bound to GroEL, however, does show a dependence upon GroEL/S concentration. The percent of tsf mutant coat protein bound to GroEL at the higher temperatures in cells with high amounts of the chaperonins was 38% and 60% for A108V and S223F, respectively, and an increase of 20–40% over the binding at normal levels of GroEL/S. Intriguingly, the percent of S223F coat protein initially bound at 30 and 30 °C increased 10–20% with the overproduction of GroEL/S, yet the level of properly folded S223F coat proteins/cell (Fig. 2) seems not to be affected by GroEL/S overproduction at these temperatures. This suggests that there are sufficient levels of assembly-competent S223F coat protein to produce phage bursts in cells with normal levels of GroEL/S, but there is some S223F coat protein that is misfolding and binding to GroEL at these permissive temperatures.

**GroEL/S Levels Have No Effect on the Rate of Release of Coat Protein from the Ternary Complex**—In order to understand the events that lead to the productive folding of coat protein in vivo, the rate of release of the coat protein from the GroEL-coat protein ternary complex was determined as described under “Experimental Procedures.” Fig. 5 shows the percent of coat protein that remained bound to GroEL after co-immunoprecipitation at various times after the addition of chase at 39 °C. The half-times calculated for the rate of release at high temperatures indicated that the tsf mutants had slower release rates than WT coat protein. For example, the release rates at 39 °C were approximately 15 s for WT coat protein and 20 and 25 s for A108V and S223F, respectively. Overall, the half-times of release decreased 6-fold for WT coat protein and 3-fold for the tsf mutants from 30 °C to 39 °C (Fig. 6). GroEL/S overproduction had no effect upon the half-times of release at any temperature, which suggests that GroEL/S overproduction rescues the phage production by increasing the probability of association between a misfolding tsf coat protein and GroEL, leading to a higher yield of assembly-competent coat proteins.

**Aggregation of Coat Protein in Vivo**—The possible fates of coat protein after being released from GroEL are assembly of procapsids, formation of inclusion bodies, or rebinding to GroEL. It is possible that, once folded, the tsf coat protein could remain in the soluble state and not assemble. However, this possibility seems unlikely since we can account for all of the newly synthesized coat protein. Gordon et al. (25) have previously shown that GroEL/S overproduction decreased the amount of tsf coat protein that has aggregated in vivo at non-permissive temperatures. That study quantified the aggregation of a single tsf coat protein mutant at only two tempera-
tures. We have conducted similar experiments but utilized different tsf mutants and examined the aggregation of coat protein over a range of temperatures (Fig. 7). From these experiments, we observed a 20% increase in the amount of aggregated WT coat protein from 30 °C to 39 °C, suggesting that WT coat protein is somewhat defective in folding at high temperatures. However, the overproduction of GroEL/S seemed not to decrease the amount of aggregation of WT coat protein at any temperature. In contrast, the tsf coat proteins aggregated in a temperature-dependent manner; increasing the temperature from 30 °C to 39 °C resulted in 40% higher amount of aggregated tsf coat proteins in cells with normal GroEL/S levels. The overproduction of GroEL/S caused a 20–30% decrease in the amount of aggregated tsf coat proteins at the higher temperatures. Interestingly, the effect of the overproduction of GroEL/S upon the aggregation of S223F coat protein becomes pronounced at a lower temperature than A108V coat protein. This result is consistent with the fact that the phenotype of A108V is less temperature-sensitive than S223F (25).

A Thermolabile Intermediate in the in Vivo Folding of the tsf Coat Protein Mutants Is Stabilized by GroEL/S—To further investigate the process by which the folding of coat protein aggregates in vivo and the effects of GroEL/S on this pathway, we performed a series of temperature shift down experiments as described under “Experimental Procedures.” The shift to a permissive temperature allows the fraction of coat protein molecules that would have partitioned down the aggregation pathway a chance to properly fold into the assembly-competent conformation. The fraction of coat protein that does not assemble into procapsids after shift down consists of those molecules incapable of assembly, which are irreversibly sequestered into inclusion bodies (25). Therefore, the rate at which the coat polypeptide chains become incapable of assembly is a measure of the lifetime of the thermolabile intermediate.

WT phage did not display any decrease in the assembly after shift down (Fig. 8). Thus, neither a shift in temperature from 39 °C to 30 °C nor the overproduction of GroEL/S had an effect on the folding of WT coat protein to the assembly-competent conformation. In contrast, the time of incubation at 39 °C in cells producing normal levels of GroEL/S had a drastic effect on the productive folding of the tsf coat protein mutants. At 39 °C, by 5 min, 50% of the tsf polypeptides were incapable of assembly, indicating the presence of a thermolabile intermediate in the folding of the tsf mutants in vivo. Increasing the amount of GroEL/S in the infected cells shifted the thermolabile folding intermediate away from the non-productive pathway since the time of incubation at 39 °C had a less deleterious effect on assembly, suggesting that GroEL/S must intercede early in the folding pathway of the tsf mutants. From these data, we conclude that, for the folding of the tsf coat proteins to be productive at high temperatures, tsf coat polypeptides must interact with a GroEL/S complex early after its biosynthesis.

The Effect of Temperature on Coat Protein Synthesis—We observed, in the experiments described above, that there was an increase in the total amount of tsf coat proteins compared
with WT coat protein synthesized in cells producing normal levels of GroEL/S with increasing temperature (Fig. 9). At 30 °C, the tsf mutants synthesized the same total amount as WT coat protein. We compared the relative amount of tsf mutant coat proteins with that of WT at 39 °C and observed a nearly 2-fold increase in synthesis. However, in cells overproducing GroEL/S, the tsf mutant coat proteins did not show this relative increase but instead synthesis remained relatively constant compared with WT coat protein. From these data we surmise that the overproduction of GroEL/S at high temperatures suppresses the increase in the amount of tsf mutant coat protein produced, perhaps by shifting the equilibrium of the thermolabile intermediate toward the productive folding and assembly pathway. Without GroEL/S overproduction, the folding of the tsf coat proteins is directed to the thermolabile, aggregation-prone state at non-permissive temperatures and the phage-infected cell compensates for this defect by increasing tsf coat protein synthesis.

**Increase in the Rate of Assembly of Coat Protein Mutants by the Overexpression of GroEL/S**—In order to observe the net effect of both productive and non-productive folding pathways in vivo, the kinetics of assembly of the coat proteins in cells producing normal or high levels of GroEL/S were examined. In Fig. 10, the incorporation of radioactive coat protein into procapsids after the addition of non-radioactive methionine/cysteine is shown. The \( t_{1/2} \) values of the assembly reactions at various temperatures were calculated as described under “Experimental Procedures.” The observed kinetics fit well to a first order reaction, although this is a simplification since the analysis of these kinetic data is difficult due to the complexity of in vivo folding and assembly. At 39 °C in cells expressing normal levels of GroEL/S, the tsf mutant coat proteins assembled more slowly than WT coat protein. S223F and A108V had \( t_{1/2} \) values of approximately 6.8 and 6.7 min, respectively, while WT assembly had a \( t_{1/2} \) of 1.5 min. When the tsf coat proteins were produced in cells with high levels of GroEL/S at 39 °C, the rates of assembly increased compared with the assembly in cells with normal amounts of GroEL/S. The \( t_{1/2} \) of assembly of S223F and A108V decreased to 3.3 and 3.1 min, respectively, upon the overproduction of GroEL/S, and the \( t_{1/2} \) of WT coat protein was 1.8 min. The variation in \( t_{1/2} \) values of the WT assembly reactions is typical of the error observed in these experiments.

The \( t_{1/2} \) values of the assembly reactions at several temperatures are shown in Fig. 11. Here, the \( t_{1/2} \) values of the assembly reactions of the tsf coat proteins were standardized to the \( t_{1/2} \) of the WT assembly reaction at each temperature to control for changes in the rate of growth. Analysis of these data revealed that the tsf mutants had relative \( t_{1/2} \) values larger than 1 in cells with normal GroEL/S levels at all temperatures, indicating that the assembly of the tsf coat proteins was slower than WT coat protein, especially at high temperatures. At temperatures above 35 °C, the rates of assembly of the tsf coat proteins slowed 3–4-fold compared with WT coat protein (rising relative \( t_{1/2} \) values). When the relative \( t_{1/2} \) of assembly of the tsf coat proteins in cells with normal levels of GroEL/S is compared with the relative \( t_{1/2} \) in cells overproducing GroEL/S at high temperatures, it is apparent that chaperonin overexpression increased the rate of assembly 2–3 fold. In cells overproducing GroEL/S at temperatures below 35 °C, the relative rate of assembly of the tsf coat proteins fell to 1.5 from a value of 2 in cells with normal GroEL/S levels. This observation is consistent with an association of the tsf coat proteins and GroEL/S even at low temperatures, as we observed in the co-immunoprecipitation experiments.

**DISCUSSION**

The bacterial chaperonins, GroEL and GroES, have been shown to stabilize misfolding polypeptides in vitro, thereby increasing the yield of the native protein by preventing aggre-
WT Coat Protein Does Not Require GroEL/S for Proper Folding in Vivo—Since the folding of WT coat protein was not significantly influenced by GroEL/S overexpression or by increasing temperatures, and plating efficiencies of WT phage were not affected in cells carrying defective GroEL or GroES, we conclude that WT coat protein does not require GroEL/S for its proper folding. In addition, the amount of WT coat protein initially bound to GroEL was not temperature-dependent and cells with overproduced levels of the chaperonins did not result in a significant increase of the amount of WT coat protein initially bound to GroEL. Nonetheless, a fraction of WT coat protein still associates with GroEL/S transiently, as both we and Gordon et al. (25) have observed. The folding of WT coat protein, however, is not affected by temperature shift down, even though we see binding to GroEL/S and WT coat protein can aggregate at high temperatures. These observations suggest an extra capacity in the amount of WT coat protein synthesized beyond what is necessary for phage biogenesis.

tsf Coat Proteins Require GroEL/S for Proper Folding and Assembly below Their Non-permissive Temperature—The tsf coat proteins assemble productively below their restrictive temperature but at a slower rate than WT coat protein, indicating that productive folding occurred. At low temperatures, normal levels of GroEL/S were sufficient to compensate for the population of tsf coat polypeptides that were misfolding. Near the restrictive temperature, however, the tsf mutants exhibited an increasing requirement for additional GroEL/S. As the temperature of cell growth is increased, the number of misfolding coat proteins is greater, and proportionately higher levels of GroEL/S must be present to restore the proper folding. Since coat protein is the major protein expressed in infected cells (25) and phage infection halts most host protein synthesis (30), it is unlikely that misfolding host cell proteins became preferential substrates of GroEL/S at higher temperatures. In fact, the misfolding tsf coat proteins are the primary species that associate with GroEL/S in the co-immunoprecipitation experiments (data not shown).

In contrast to WT coat protein, the ability of the tsf coat proteins to form capsids in cells that carry defective GroEL or GroES was reduced even at permissive temperatures, indicating a distinct requirement for basal levels of GroEL/S regardless of temperature. This finding was consistent with the fact that substantial amounts of tsf coat protein were initially bound to GroEL at low temperatures. The amount of S223F coat protein bound to GroEL increased when the GroEL concentration was raised regardless of temperature, suggesting that even at low temperatures the improper folding of S223F coat protein exceeded the capacity of GroEL to accommodate substrates. However, at these low temperatures, there was no reduction in the amount of S223F coat protein aggregated and the amount of properly folded S223F coat proteins/cell did not increase with higher levels of the chaperonin, suggesting that there is more coat protein synthesized than is needed for phage biogenesis. A108V did become GroEL/S-dependent at a higher temperature than did S223F, indicating that A108V coat protein folding and assembly is not as defective as that of S223F.

Protein Synthesis Helps Compensate for Defective Folding—During these studies, we noted a distinct increase in the total amount of tsf coat protein synthesized at higher temperatures when compared with WT coat protein. This increase occurred only in cells producing normal levels of GroEL/S. We hypothesize that the infected cell, in order to produce the same number phage, attempts to keep the pool of assembly-competent tsf coat protein monomer constant with increasing temperature, despite aggregation. Eventually, in cells with normal levels of GroEL/S, a temperature is reached where the partitioning of the majority of the tsf coat protein is shifted to the non-productive aggregation pathway. GroEL/S overproduction shifts the partitioning of tsf coat protein back toward the productive folding pathway even at high temperatures, so that increased tsf coat protein synthesis is not necessary for the cell to produce normal phage bursts.

Assembly of P22 Coat Protein in Vivo—Analysis of the assembly of P22 coat protein in vivo revealed that the tsf coat proteins have slower assembly rates at higher temperatures, suggesting that there was a decrease in the concentration of assembly-competent monomer. Several experiments presented herein suggest that 33–35 °C may represent a critical temperature range at which GroEL/S approached substrate saturation and the GroEL/S-coat protein turnover rate was not suffi-
The cellular concentration of GroEL/S resulted in an increased amount of tsf coat proteins that achieve the assembly-competent state. In addition, increasing GroEL/S concentration led to increases in the ratio of assembly of the tsf coat proteins at low temperatures, demonstrating the existence of a slightly defective of the tsf coat protein molecules that was not compensated for at normal GroEL/S levels. The assembly rates of the tsf coat proteins, however, even with GroEL/S overproduction at low temperature, are still slower than WT coat protein. These data support the notion that tsf mutant coat proteins are also somewhat defective in assembly, rather than exclusively defective in their folding. We support this view since GroEL/S levels are sufficiently high upon overexpression to compensate for the folding defect at low temperatures, and consequently no difference between assembly rates of WT and tsf coat proteins should be observed. Thus, we conclude that the slower assembly rates of the tsf coat proteins is a result of two components: a folding defect and an alteration in the conformation of the folded monomer. At low temperatures, the conformational defect is primarily responsible for the change in assembly rates, whereas at higher temperatures, defective folding makes the largest contribution to the overall decrease in assembly rate. This is consistent with in vitro results (13, 15).

Model of the Folding Pathway of the tsf Coat Proteins of Phage P22 in Vivo—The in vivo folding pathway of the tsf coat proteins at permissive and non-permissive temperatures along with the overproduction of GroEL/S is summarized in Fig. 12. At low temperatures, the flux of tsf coat protein is poised toward the productive folding pathway, which leads to assembly of procapsids. GroEL/S levels are sufficient to cause the productive folding of a relatively low number of misfolding tsf coat proteins at these temperatures. At high temperatures with normal levels of GroEL/S, the flux of tsf coat protein is shifted to the non-productive aggregation pathway and ultimately results in the formation of inclusion bodies. Here, the chaperonin levels are not sufficient to keep the flux of tsf coat protein toward the assembly pathway. As a result, the amount of tsf coat protein in the assembly-competent state in the soluble fraction is low because of increased levels of aggregation and inclusion body formation. The phage-infected cell responds to the lack of assembly-competent monomers in the cytoplasm by increasing tsf coat protein synthesis. Raising the intracellular concentration of GroEL/S suppresses aggregation by shifting the equilibrium between the folding intermediate (I) and the thermolabile intermediate (I*) back to the productive pathway. As a result, the amount of assembly-competent monomers in the soluble fraction increases, shifting the net flux of tsf coat protein to the productive pathway, and the cell responds by synthesizing normal amounts of tsf coat protein.

The model that we propose accounts for the overall flux of the tsf proteins within the cell, but does not address how GroEL/S overexpression rescues tsf coat protein assembly. The requirement for the overexpression of GroEL/S for the rescue of tsf coat protein folding and assembly at high temperatures arises as a result of an inefficiency of GroEL in the recognition of the folding of the tsf coat proteins rather than saturation of GroEL with tsf coat proteins. If we consider a cell producing normal levels of GroEL/S, a normal phage burst produces ~100 phage/cell in 30 min (data not shown); thus, if we assume a constant rate of synthesis, there are ~1,400 coat proteins produced/min/cell at low temperature, and 2,800 tsf coat proteins produced/min/cell at high temperatures considering the 2-fold increase in tsf coat protein synthesis. Using estimates of GroEL levels within E. coli (41), we have calculated, that in Salmonella, which produces similar amounts of GroEL/S as E. coli (data not
shown), there are ~3000 molecules of GroE/phage infected cell and overproduced levels increase this amount to approximately 14,000 molecules of GroE/phage infected cell (25, 41). Since each coat protein interacts with GroEL for less than 1 min at high temperature, there are sufficient levels of GroEL and GroES for complex formation with the tsf coat proteins synthesized even at low concentrations of the chaperonins. We conclude that the encounter between GroEL and the tsf coat proteins at high temperatures is not an efficient process and that the rate of aggregation of the tsf coat proteins must exceed the rate of interaction with GroEL/S at normal concentrations. Therefore, increasing the concentration of GroEL/S within the cell alleviates this inefficiency of recognition of an aggregation prone tsf coat protein by GroEL by enhancing the likelihood of interaction with the chaperonins before irreversible aggregation can occur.

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