Selective in Vivo Rescue by GroEL/ES of Thermolabile Folding Intermediates to Phage P22 Structural Proteins*

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The in vivo conformational substrates of the GroE chaperonins have been difficult to identify, in part because of limited information on in vivo polyepitide chain folding pathways. Temperature-sensitive folding (tsf) mutants have been characterized for the coat protein and tailspike protein of phage P22. These mutations block intracellular folding at restrictive temperature by increasing the lability of folding intermediates without impairing the stability or function of the native state. Overexpression of GroEL/ES suppressed the defects of tsf mutants at 17 sites in the coat protein, by improving folding efficiency rather than assembly efficiency or protein stability. Immunoprecipitation experiments demonstrated that GroEL interacted transiently with newly synthesized wild-type coat protein and that this interaction was prolonged by the tsf mutations. Folding defects of the tailspike polypeptide chains were not suppressed. A fraction of the tsf mutant tailspike chains bound to GroEL but were inefficiently discharged. The results suggest that 1) thermolabile folding intermediates are natural substrates of GroEL/ES; 2) although GroEL may bind such intermediates for many proteins, the chaperoning function is limited to a subset of substrate proteins; and 3) a key reason for the heat-shock response may be to stabilize thermolabile folding intermediates at elevated temperatures.

The Escherichia coli genes GroEL and GroES were discovered in screens of mutagenized bacteria for loss of ability to propagate bacteriophage (Georgopoulos et al., 1972; Takano and Kakefuda, 1972; Coppo et al., 1973; Sternberg, 1973). These two host genes constitute a single operon whose expression is increased as part of the bacterial heat-shock response (Hemmingsen et al., 1988). Homologs of these proteins, referred to as Hsp60 and Hsp10, have been found in mitochondria and chloroplasts (Hemmingsen et al., 1988; Cheng et al., 1989; Hartman et al., 1992; Bercht et al., 1992). A hetero-oligomeric ring found in the eukaryotic cytosol, which contains the TCP-1 protein, can mediate the folding of several proteins in an ATP-dependent manner. A fraction of the tsf mutant tailspike chains bound to GroEL but were inefficiently discharged. The results suggest that 1) thermolabile folding intermediates are natural substrates of GroEL/ES; 2) although GroEL may bind such intermediates for many proteins, the chaperoning function is limited to a subset of substrate proteins; and 3) a key reason for the heat-shock response may be to stabilize thermolabile folding intermediates at elevated temperatures.

Most Hsp60s function as 14-mers consisting of two stacked heptameric rings, while Hsp10 is a single ring of 7 subunits (Hendrix, 1979; Zwickl et al., 1980; Hartman et al., 1992). GroEL/ES are essential for E. coli viability at all temperatures (Fayet et al., 1989). Hsp60/Hsp10 have been shown to mediate the folding of a number of proteins, by preventing aggregation arising from incompletely folded molecules (Vitanen et al., 1991; Gething and Sambrook, 1992; Hendrick and Hartl, 1993). In the case of oligomeric proteins, it is not known if GroEL/ES also mediate protein assembly.

Hsp60 can bind to a diverse set of partially folded protein substrates but not to their native states (Martin et al., 1991; van der Vies et al., 1992; Vitanen et al., 1992). Folding probably occurs in the central cavity of Hsp60, so that discharged substrate molecules are released in a conformation different (often committed to correct folding) from that of incoming substrate molecules (Martin et al., 1991; Langer et al., 1992; Braig et al., 1993). Folding generally occurs in an ATP-dependent manner and requires cycles of binding and release of Hsp10 (Martin et al., 1993a). Molecular chaperone proteins can function cooperatively, with Hsp70 proteins recognizing extended polypeptides which are then transferred to Hsp60 (Cheng et al., 1989; Kang et al., 1990; Manning-Krieg et al., 1991; Langer et al., 1992a). Hsp60 has been shown to reduce the extent of denaturation of native proteins (Martin et al., 1992). However, Horwich et al. (1993) have reported that a temperature-sensitive lethal mutation in GroEL did not cause destabilization of prefolded proteins. It has been difficult to establish whether or not folding intermediates, which may be particularly sensitive to elevated temperatures, are the major substrates of Hsp60 in vivo. Van Dyk et al. (1989) have reported that overexpression of GroEL/ES can rescue a subset of temperature-sensitive mutations in Salmonella and P22. Here we have taken advantage of the availability of well defined sets of temperature-sensitive folding mutants of two phage P22 structural proteins to probe chaperonin function in vivo. The tsf mutations act by further destabilizing an intracellular folding intermediate, without altering the stability of the native state (Goldenberg et al., 1988; Sturtevant et al., 1989).

The P22 tailspike protein, encoded by gene 9, is a trimer, each chain of which is 666 amino acids and has a molecular mass of 72 kDa (Sauer et al., 1982). The tailspike is a structural protein of the phage head which is involved in host recognition. Its structure is dominated by a β-helix motif (Gleibach et al., 1984). Temperature-sensitive folding mutants of the tailspike protein destabilize a single chain folding intermediate without destabilizing the native folded trimer (Goldenberg and King, 1981; Goldenberg et al., 1988; Sturtevant et al., 1989). At elevated temperatures, single chain intermediates transiently accumulate and then aggregate into inclusion bodies (Haase-Pettingell and King, 1988).

1 The abbreviations used are: tsf, temperature-sensitive folding; ts, temperature-sensitive.
The P22 coat protein, encoded by gene 5, is composed of 430 amino acids and has a molecular mass of 47 kDa (Eppler et al., 1991). 420 icosahedrally arranged coat protein molecules are the major protein constituents of phage heads, within which is located P22 DNA (King and Casjens, 1974). A set of 18 temperature-sensitive mutants, at 17 different sites in the coat protein, have been characterized. Phage particles and precursor phage particles (procapsids) carrying these mutations were not destabilized at restrictive temperature in vivo (Gordon and King, 1993). In vitro, the stabilities of folded but unpolymerized mutant chains, and of mutant procapsids, were similar to their wild-type counterparts. In vivo, mutant coat protein synthesized at restrictive temperature was not degraded and instead accumulated as inclusion bodies (Gordon and King, 1993). The mutants chains are recessive to wild-type, do not exhibit intragenic complementation, and cannot be rescued by wild-type subunits. These genetic properties are also consistent with their being defective in the folding process (Gordon and King, 1994). These results indicate that these temperature-sensitive mutants of the coat protein, like the tailspike mutants, are defective in folding.

Similar folding mutations have been reported for luciferase (Sugihara and Baldwin, 1988), lactate dehydrogenase (Truong and Baldwin, 1988), lactate dehydrogenase (Truong et al., 1989), and interleukin 1β (Chrunyk et al., 1991), and  interleukin 1β (Chrunyk et al., 1991), and  interleukin 1β (Chrunyk et al., 1991). 420 icosahedrally arranged coat protein molecules are located P22 mutant chains, and of mutant procapsids, were similar to their genic complementation, and cannot be rescued by wild-type (Sugihara and Baldwin, 1988), lactate dehydrogenase (Truong and Baldwin, 1988). Similar folding mutations have been reported for luciferase (Sugihara and Baldwin, 1988), lactate dehydrogenase (Truong and Baldwin, 1988), lactate dehydrogenase (Truong et al., 1989), and interleukin 1β (Chrunyk et al., 1991), and  interleukin 1β (Chrunyk et al., 1991). These results indicate that these temperature-sensitive mutants of the coat protein, like the tailspike mutants, are defective in folding.

Growth Curves—Exponentially growing DB7136/pBR322 and DB7136/pOF39 growing in Minimal/Amp were diluted 1:50 in fresh LB/Amp grown to a concentration of 1 x 109/ml, were infected, pelleted, resuspended in fresh media to a concentration of 4 x 109/ml and incubated at 37°C. At 20 min after infection, cells were labeled with 25 µCi/ml 35S-amino acids (to 2%). At the indicated times, 700 µl of infected cells were added to 78 µl of buffer A (500 µM Tris-HCl, 1 mM NaCl, 100 µM EDTA, pH 7.6) and immediately frozen in dry ice/ethanol bath. Cells were thawed at 4°C, where all subsequent manipulations were performed.

Polyclonal Anti-E. coli GroEL, obtained from rabbits, and corresponding preimmune sera, were the gifts of Caroline Donnelly and Graham Walker (Donnelly and Walker, 1992). Antibodies were diluted 1:20 in buffer B (50 mM Tris-HCl, 100 mM NaCl, pH 7.6). Protein A-Sepharose beads (Pierce), 20% in buffer C (buffer B with 0.5% Triton X-100 and 0.25% Nonidet P-40), were mixed 1:1 with antibodies, rocked for 1 h, centrifuged in a microcentrifuge for 1 min, washed with buffer D (buffer B with 0.25% Triton X-100 and 0.25% Nonidet P-40), rocked for 1 h, and centrifuged for 1 min. The bead/antibody pellet was resuspended to 10% beads in buffer D.

Thawed infection samples were centrifuged in a microfuge for 5 min. Supernatants were carefully removed and resupernatants were mixed 3:2 with the beads/antibodies, followed by rocking for 1 h. Samples were centrifuged for 30 s and washed in one-tenth buffer A. Pelleted beads were boiled in SDS sample buffer, electrophoresed through SDS gels, and exposed to film.

PhosphorImager Quantifications—Coat protein, tailspike protein, GroEL, DnaK, and gpl were quantified by exposing gels to PhosphorImager screens and using the ImageQuant software (Molecular Dynamics). Bands were circled using the "region" tool at x4 magnification. Background was defined as counts/pixel in each lane immediately above DnaK, where no protein bands were visible.

**RESULTS**

**Effect of GroEL/ES Overexpression on Plating Efficiencies of tsf Mutant P22 Phage**—The E. coli GroES operon, consisting of the natural GroES and GroEL genes, was cloned into a pBR322-derivative plasmid (Fayet et al., 1986). This plasmid, called pOF39, was transferred into S. typhimurium, the host for phage P22. The ability of pOF39 to rescue temperature-sensitive folding alleles of the P22 coat protein and tailspike protein genes was examined.

Phage were plated with Salmonella cells carrying either the pOF39 plasmid or a control plasmid. For each phage strain, the ratio of plaques seen at several test temperatures to plaques seen at 24°C with control cells was determined. Wild-type phage exhibited almost no decrease in plating efficiency, as
The relative plating efficiencies of 15 tsf tailspike protein strains were also determined (Fig. 1C). In marked contrast to the results for the coat protein strains, overexpression of the GroE operon had no effect on the plating efficiency of any of the temperature-sensitive tailspike strains examined.

Effect of GroEL/ES Overexpression in Liquid Culture—The kinetics and extent of phage propagation was quantitatively examined by performing one-step growth curve experiments with and without GroEL/ES overexpression. Growth curves were performed at 28 and 37 °C with wild-type phage and three tsf coat protein strains (Fig. 2A). These temperature-sensitive strains are restricted for growth at ~37 °C, as assayed by plating. Wild-type phage had a burst of ~270 phage/cell at 28 °C, on both pOF39 cells and control cells, and a burst of ~350 phage/cell at 37 °C with both cell types. At 28 °C the three tsf coat strains had bursts which were about 75% of the wild-type burst. At 37 °C all three ts coat strains had bursts of less than one phage/cell on control cells. Bursts at 37 °C for the coat ts mutants incubated with the pOF39 cells were ~500 times higher than with the control cells. The pOF39

assayed by number of plaques, on either host, up to 41 °C (Fig. 1A), although some reduction in plaque size was observed.

Phage carrying the tsf coat protein and tailspike alleles exhibit sharp reductions in plating efficiencies at elevated temperatures on wild-type cells (Smith et al., 1980; Gordon and King, 1993, 1994). At their minimum restrictive temperatures of growth, most of these strains have reductions in titer of about 10^6, which is in the range of that expected for reversion events.

The minimum restrictive growth temperature was significantly increased for each of the 18 tsf coat protein strains when plated on the pOF39 cells (Fig. 1B). For example, the extremely temperature-sensitive strains 5Ts(Pro310Ala) and 5Ts(Thr294Ile) were rescued at 33 °C, while the least temperature-sensitive strains 5Ts(Tyr411His), 5Ts(Trp48Gln), and 5Ts(Gly403Asp) were rescued at 41 °C. Plaque counts with pOF39 cells were increased to 24 °C levels and were about 10^6 higher than with control cells. At temperatures below the minimum restrictive growth temperature, plaques were larger on the pOF39 cells than on control cells.

The relative plating efficiencies of 15 tsf tailspike protein strains were also determined (Fig. 1C). In marked contrast to the results for the coat protein strains, overexpression of the GroE operon had no effect on the plating efficiency of any of the temperature-sensitive tailspike strains examined.
FIG. 2. Kinetics of phage growth. Exponentially growing pOF39 cells or pBR322 cells were infected with wild-type phage, tsf mutant coat protein phage, or tsf mutant tailspike phage at 37°C (A) or 28°C (A) or 30°C (B) 12 min after infection. Portions were shifted down to 28°C (A) or 30°C (B) 12 min after infection. At the indicated times samples were withdrawn, lysed with CHCl₃, and titrated. A and B were each independent experiments.

37°C burst was about 20% of the corresponding wild-type burst for 5Ts(Ser262Phe), 35% for 5Ts(Asp174Asn), and 60% for 5Ts(Ser223Phe). At 37°C the kinetics of phage growth on the pOF39 cells were slightly delayed in the mutants as compared with wild-type phage.

Growth curves performed at 30 and 39°C with wild-type phage, the coat protein mutant 5Ts(Pro418Ser), and the two tailspike mutants 9Ts(Thr307Ala) and 9Ts(Gly244Arg) are presented in Fig. 2B. On plates, 5Ts(Pro418Ser) was restricted for growth at ~37°C, and the two tailspike mutants were partially restricted at 39°C and completely restricted at 41°C. Wild-type phage yielded ~350 phage/cell at 30°C and ~400 phage/cell at 39°C. At 39°C 9Ts(Thr307Ala) yielded ~one phage/cell on both cell types and 9Ts(Gly244Arg) yielded ~three phage/cell. At 39°C, the coat tsf mutant 5Ts(Pro418Ser) yielded less than one phage/cell on control cells, while it had achieved about 25 phage/cell on the pOF39-carrying cells 120 min after infection. This coat ts rescue was less than that demonstrated in Fig. 2A because of the higher temperature. Even though 39°C is ~2 degrees above the restrictive growth temperature for 5Ts(Pro418Ser), the ts coat protein mutant was significantly rescued by the GroE overexpression while the tailspike mutants exhibited no improvement in propagation efficiency.

Requirement for GroES Overexpression—Is overexpression of both GroE proteins required for rescue of the coat protein mutants? For these experiments Salmonella cells carrying pTG10-derivative plasmids were used. pLS carried the GroE operon cloned directly from pOF39 into pTG10. A frameshift mutation in the GroES gene was introduced to generate pL. It has been reported that this mutation was not polar on expression of the downstream GroEL gene (Goloubinoff et al., 1989). pS carries the natural GroE promoter, followed by GroES and only the first 30% of the GroEL coding sequence (Van Dyk et al., 1989; Fayet et al., 1986). Control cells carried only the pTG10 plasmid without the GroE operon. The efficiency of plating of the tsf coat protein and tsf tailspike protein strains was examined on these four cell types at permissive temperature and at each strain’s particular minimum restrictive temperature.

Wild-type phage exhibited no reduction in plating ability at 30°C and 41°C on all four cell types (Table I). All 18 tsf coat
protein strains formed plaques with high efficiency at 30°C on all four cell types. At their specific restrictive temperatures, the efficiency of plating on pTG10 cells, pS cells, and pL cells was sharply reduced. In contrast, on the pLS cells, plating efficiency increased to near permissive levels. These results demonstrated that overexpression of both GroEL and GroES was required for rescue of the ts coat protein strains. The results also illustrated that the rescue could be attained with two different plasmid systems, as long as the GroE operon was present. Though still substantial, the rescue achieved with pLS was reproducibly slightly less than that achieved with pOF39. This is consistent with the expected lower copy number of the pLS plasmid (Sambrook et al., 1989).

On all four cell types, the ts tailspike strains plated with high efficiency at permissive temperature and low efficiency at restrictive temperature (Table I).

**Effect of GroEL/ES Overexpression on Protein Folding**—Newly synthesized tsf coat protein and tailspike protein accumulate in inclusion bodies at restrictive temperature (Haase-Pettingell and King, 1988; Gordon and King, 1993). To follow the fate of newly synthesized protein in the presence or absence of GroE overexpression, pOF39 cells and pBR322 control cells grown in minimal media were infected at temperatures ranging from 29 to 39°C, with wild-type phage, with 5Ts(Phe353Leu), or with 9Ts(Gly244Arg). In this experiment phage strains also carried a gene 3 amber mutation, which blocks prokaryotic phage assembly at the procapsid stage (Botstein et al., 1973). Infected cells were pulsed with [14C]aminoc acids and incubated for 35 min. Cultures were subjected to freezing followed by thawing at room temperature, twice, which gives efficient cell lysis (Haase-Pettingell and King, 1988; Gordon and King, 1993).

A pellet/supernatant separation was then performed. Inclusion bodies are pelleted in this procedure, while procapsid particles and native tailspike trimers remain in the supernatant (Haase-Pettingell and King, 1988; Gordon and King, 1993). Wild-type coat protein remained largely in the supernatant, presumably assembled into procapsid particles, at all temperatures tested on both cell types (Fig. 3A). In contrast, the 5Ts(Phe353Leu) coat polypeptide chains were largely pelleted at the higher temperatures when control cells were infected. When the infection of 5Ts(Phe353Leu) phage was performed on pOF39 cells, the percentage of pelleted coat protein was sharply reduced at all temperatures. These results establish that GroE overexpression is improving coat protein folding efficiency by significantly reducing the levels of coat protein inclusion body formation.

Tailspike folding efficiency was assayed by determining the distribution of tailspike between native trimers and incompletely folded species. Native trimers are resistant to solubilization by SDS, while inclusion body aggregates and partially folded species are solubilized by SDS (Haase-Pettingell and King, 1988). As shown in Fig. 3B, wild-type tailspike folded and assembled into trimers with high efficiency at temperatures up to 35°C on both cell types examined. At 39°C the efficiency of trimerization was reduced, and this was not improved by overexpression of GroE. The tsf tailspike mutant 9Ts(Gly244Arg) exhibited reduced folding efficiency at temperatures of 33°C and higher, which was not improved by GroE overexpression.

**Induction of the Heat-shock Response by tsf Mutants**—The levels of GroEL and DnaK, which are major prokaryotic heat-shock proteins, were quantified from SDS gel lanes corresponding to the 39°C lysates described above (Fig. 4). These samples had been labeled with [14C]-amino-acids 45 min after infection. On the pOF39 cells, the levels of GroEL synthesis in the infections with the coat and tailspike tsf mutants were ~2-fold higher than in the wild-type infection (Fig. 4B). GroEL synthesis was also induced in the control cells. In addition, DnaK synthesis was induced on both cell types by the ts mutants (Fig. 4C). Apparently, the incorrectly folded coat and tailspike protein were able to serve as signals for augmenting the heat shock response.

Fig. 4 also demonstrates that the pOF39 plasmid, which rescued the coat tsf mutants, generated a ~9-fold increase in the rate of GroEL synthesis at 39°C, when infected with 5Ts(Phe353Leu) or 9Ts(Gly244Arg), as compared with control cells.

**Kinetics of in Vivo Interaction with GroEL**—Immunoprecipitation of phage-infected cell extracts with anti-GroEL antibodies was performed to study the in vivo interaction between GroEL and the coat and tailspike proteins. The pOF39 cells were grown in minimal media and infected at 37°C with wild-type phage, 5Ts(Ser223Phe), or 9Ts(Ser223Phe). 50 min after infection, cultures were pulse-labeled with [14C]-aminoc acids and chased 30 s later. Samples were withdrawn and immunoprecipitated with anti-GroEL antibodies or control serum at various times after labeling. Anti-GroEL precipitates and control samples were electrophoresed through SDS gels and exposed to
films, shown in Fig. 5, A-C. Quantification of coat and tailspike protein bound to GroEL, from each of the three infections, is presented in Fig. 5, D-F.

With wild-type phage (Fig. 5D), 0.75 min after labeling, 25% of coat protein was bound to GroEL, and 2 min after labeling, 5% was bound. For about 6 min after labeling, ~7% of tailspike protein was bound. This suggested that the majority of tailspike protein did not bind GroEL, and once formed, the complex decayed slowly.

With 5Ts(Ser223Phe) phage (Fig. 5E), 0.75 min after labeling 43% of coat protein was bound to GroEL, and 2 min after labeling 21% of the coat protein was bound. 5Ts(Ser223Phe) coat protein transiently interacted with GroEL and was discharged at a substantially slower rate than wild-type coat protein. The amount of bound tailspike protein in the ts coat protein infection was reproductively reduced as compared with tailspike bound in the wild-type phage infection, even though the levels of GroEL were ~2-fold higher, as if ts mutant coat protein were saturating available GroEL.

With 9Ts(Thr235Ile) phage (Fig. 5C), the (wild-type) coat protein had similar binding behavior to GroEL as seen with wild-type phage. At early times after labeling, 30% of ts mutant tailspike protein was bound to GroEL, and this complex decayed slowly, with an apparent half-life of ~35 min. Extrapolating toward early times based on the slow rate of decay suggests that most ts mutant tailspike protein did not interact with GroEL.

By mass, ~15 times more coat protein was synthesized than tailspike protein. This binding of ts tailspike protein to GroEL may be counterbalanced by the ~2-fold increase in the rate of GroEL synthesis, so that similar amounts of GroEL remained free for binding to coat protein.

At the 2-min time points, there was ~50% more labeled coat protein in the lysates than at the 0.75-min time points, pre-
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sumably due to completion of labeled nascent chains after addition of cold amino acids. This suggests that the initial rate of coat protein-GroEL complex discharge is faster than that plotted and that most newly synthesized coat protein chains may have undergone a brief interaction with GroEL.

Can GroEL/ES Overexpression during Particle Formation Improve Stability?—Phage particles carrying the tsf coat protein mutations are not destabilized at restrictive temperature in vivo (Gordon and King, 1993). However, phage particles from the two strains 5Ts(Tyr48Gln) and 5Ts(Gly403Asp) are less stable than wild-type particles, when incubated in a destabilizing medium lacking Mg\(^{2+}\). Thus, in addition to the folding defect which confers the ts phenotype, these two mutations also result in less stable assembled phage particles. Stocks of these two strains grown on cells overexpressing GroEL/ES were prepared. As shown in Table III, these phage exhibited the same reduction in titer when incubated in medium lacking Mg\(^{2+}\) as phage grown on control cells. As shown above, GroEL/ES overexpression could improve folding efficiency, but in this case such overexpression did not result in more stable particles.

Interestingly, these two mutants are among the least restricted for growth in vivo, and yet the other coat protein ts phage (such as 5Ts(Thr249Ile)), which have lower restrictive growth temperatures (Gordon and King, 1994), are not destabilized under these in vitro conditions. Thus, in agreement with the in vitro studies, the stability of phage particles, and therefore of the folded coat protein molecule, is the not the key factor in determining the in vivo temperature sensitivity. The mutations appear to differentially destabilize folding intermediates and the folded protein (Gordon and King, 1994).

**DISCUSSION**

Folding intermediates are generally less structured than their own native state and, therefore, capable of destabilization to destabilization (Haase-Pettingell and King, 1988; Mitraki and King, 1989; Viitanen et al., 1990; Goldberg et al., 1991; van der Vies et al., 1992). Cells infected at restrictive temperature with tsf mutants of the coat and tailspike proteins transiently accumulate thermolabile folding intermediates (Goldenberg et al., 1983; Gordon and King, 1993; Danner and Seckler, 1993). A key reason for the heat-shock response may be to stabilize folding intermediates at elevated temperatures.

GroEL/ES Overexpression Rescues Coat Molecules with Folding Defects—Overexpression of GroEL/ES rescued the tsf coat protein mutants by sharply reducing the levels of aggregating newly synthesized coat chains. The rescue of coat protein folding mutants reported here identifies a class of in vivo GroES substrates. Since the coat protein ts mutants are defective in folding, the GroEL/ES proteins are functioning by improving the folding efficiency rather than the assembly efficiency or stability of this oligomeric protein. For the thermolabile class of ts mutations, which act by destabilizing the native state, rescue of ts mutants may proceed through interacting with unfolding intermediates, resulting in apparent stabilization of the native state (Martin et al., 1992).

At late times after infection, the coat polypeptide chain is the major species being synthesized in the infected cell. Presumably, the endogenous levels of GroEL/ES are inadequate to cope with the intracellular concentration of mutant coat protein folding intermediates. The GroEL levels in the cells harboring the pOF39 plasmid were nearly 10-fold higher than in cells harboring the control plasmid.

The tsf mutations may prolong the conformation of a folding intermediate which is recognized by GroEL or lead to an altered conformation, due to destabilization of a wild-type folding intermediate, which is recognized by GroES (Haase-Pettingell and King, 1985). A model for the action of temperature-sensitive folding mutations posits an equilibrium between the productive folding intermediate and an off-pathway species (I*-1). Elevated temperatures result in increased levels of I*, which is the species that has the property of aggregating (Haase-Pettingell and King, 1988; Mitraki and King, 1989). Tsf substitutions further shift the equilibrium to I'. If chaperonins recognize I', they could block aggregation, in this case without participating in the productive pathway. The mutants may disrupt a critical conformation yielding a common off-pathway intermediate (I').

![Fig. 4. Rates of GroEL and DnaK synthesis.](image-url)
Fig. 5. Immunoprecipitations of phage-infected cells with anti-GroEL. A and D, wild-type. B and E, 5T(Ser233Phe). C and F, 9T(Thr235Ile). Exponentially growing pOF39 cells were infected at 37 °C. Infections were labeled with 3H-amino-acids and chased 30 s later. Samples were removed at the indicated times after labeling. These were mixed with buffer A, frozen in dry ice/ethanol, and thawed at 4 °C, where subsequent manipulations were performed. 14 µl of each sample were removed (lysate; lane 1). The remaining were subject to two prespins. Pellets, representing un-lysed cells and aggregated material, were resuspended in SDS sample buffer to the volume of the supernatants, and 14 µl was loaded on the gels (prespin pellet; lane 2). 14 µl of supernatant fractions were removed (prespin supernatant; lane 3). 300 µl of each supernatant was immunoprecipitated with preimmune serum (lane 4) or anti-GroEL antibodies (lane 5). Samples were mixed with SDS sample buffer, boiled, electrophoresed through 10% SDS gels, and exposed to films. Quantification of the amount of coat protein and tailspike protein bound to GroEL was performed with a PhosphorImager (“Experimental Procedures”). The amount of specifically immunoprecipitated coat, tailspike, and GroEL was computed by subtracting counts in the control immunoprecipitation (lane 4) from corresponding counts in the anti-GroEL immunoprecipitation (lane 5). This value, divided by the levels of corresponding protein present in the original lysates (determined from lane 1), gave the percent of immunoprecipitated protein. The values presented in D, E, and F are normalized to the efficiency of immunoprecipitation of GroEL for each time point. (% immunoprecipitated coat or tailspike)/(% immunoprecipitated GroEL).

Recognition of such a common intermediate would rescue the diverse starting mutants, which were present at 17 different sites (Gordon and King, 1993).

Tsf mutants of the tailspike protein were not rescued by GroE overexpression. These mutations generate thermolabile intermediates in the tailspike folding pathway (Smith et al.,...
The tailspike is a large trimeric protein whose secondary structure is dominated by the recently discovered parallel β-coil motif (Steinbacher et al., 1994). The folding and assembly of the coat protein have been studied in vitro (Prevelige et al., 1988; Teschke and King, 1993; Teschke et al., 1993), although its structure is not yet known. Different chaperones may be specialized for classes of protein conformations. Alternatively, the 72-kDa tailspike protein may be too large to permit productive folding within the GroEL ring (Langer et al., 1992b). Similarly, folding steps occurring after the assembly of protein oligomers, which may be too large for GroEL, may not be well chaperoned.

Interaction of Folding Intermediates with GroEL—The recovered complexes of protein bound to GroEL were stable for several hours without the use of cross-linking agents. The CDTA in the lysis buffer was expected to chelate Mg²⁺ ions necessary for ATP hydrolysis (Martin et al., 1991). In addition, the effective concentrations of GroES and ATP, which are generally involved in complex discharge, were reduced upon cell lysis.

At the earliest time point, a significant fraction of the labeled wild-type coat chains were immunoprecipitated with anti-GroEL. However, from the data reported here, it is not clear if this interaction was essential for productive folding. It appears that nearly all 5Ts(Ser223Phe) coat protein transiently interacted with GroEL. When bound to GroEL, 5Ts(Ser223Phe) coat protein required more time to achieve the conformation permitting final release and presumably committed to assembly. It is possible that 5Ts(Ser223Phe) coat protein cycled between a bound state and a prematurely released state. This reduced rate of GroEL turnover may explain the requirement for the higher concentrations of GroEL/ES necessary to mediate the folding of the ts mutant coat protein.

Less than 10% of wild-type tailspike protein was recovered bound to GroEL, and this bound protein had a slow rate of discharge. Apparently, most wild-type tailspike protein does not pass through a conformation which is recognized by GroEL while folding in vivo; and the tailspike protein that does bind is released extremely slowly. 30% of 9TsfThr235Ile tailspike was discharged with a half-time of ~35 min, apparently without reaching the native state. The tailspike protein could also have undergone periods of release followed by re-binding. It is possible that upon temperature reduction in vivo, tsf mutant tailspike protein which had bound to GroEL at high temperature could be released in a conformation permitting correct folding (Holl-Neugebauer et al., 1991). In this experiment, ~70% of tsf mutant tailspike protein aggregated without being bound by GroEL.

Van Dyk et al. (1989) reported that several temperature-sensitive proteins in S. typhimurium, E. coli, and the phage P22 tailspike protein could be suppressed by overexpressing GroEL/ES. Such suppression was reported to occur for four of nine tsf tailspike mutants examined. In their experiments on 9Ts(Gly424Arg), GroEL/ES overexpression increased plating efficiency at 39°C from less than $10^{-4}$ to 0.1 and bursts from 0.2 to 3% of the permissive value (Van Dyk et al., 1989). Comparison with the much greater increases in yields seen for the coat ts mutants suggests that these responses do not represent significant rescue. The results reported in our work confirm the low level of rescue of the tsf tailspike mutants, as seen by several assays. We conclude that overexpression of GroEL cannot suppress any of the tsf tailspike mutants.

Brunschier et al. (1993) have examined the interaction between purified wild-type tailspike protein and GroEL/ES in vitro. They found that GroEL/ES could not significantly improve the folding efficiency of the wild-type tailspike protein, after denaturation by acid urea. GroEL formed a complex with refolding wild-type tailspike protein at high temperature (above 30°C). This complex could be discharged with addition of ATP, but at high temperature the tailspike protein was discharged in a conformation still susceptible to aggregation. The amount of bound tailspike was reduced on subjection of a preformed complex to temperatures below 25°C, suggesting that the tailspike was not tightly bound.

Uncoupling of GroEL Binding from Chaperoning of Folding—The results reported here, together with those of Brunschier et al. (1993), demonstrate that GroEL can bind tailspike folding intermediates without efficiently chaperoning folding, and thus that substrate binding and chaperoning of folding are separate aspects of GroEL function. These results indicate that GroEL/ES do not mediate folding only through binding to and sequestering substrates. These results are consistent with previous evidence that, in productive interactions, substrates fold to a non-aggregating conformation while complexed with GroEL (Martin et al., 1991).

Several studies have been performed to examine the substrate binding determinants of Hsp60 and Hsp70 chaperones (Landry and Gierasch, 1991; Flynn et al., 1991; Landry et al., 1992; Viitanen et al., 1992). Since binding alone is not sufficient for productive chaperone function, extrapolation from binding data to substrate characterization requires caution. It will be useful to differentiate between substrates whose folding can be improved by the chaperones and those whose folding cannot be improved.

Necessity of GroES—The presence of Hsp60/Hsp10 leads to a much improved yield of folding for rhodanese (Martin et al., 1991; Mendoza et al., 1991), Rubisco (Goloubinoff et al., 1989), and citrate synthase (Buchner et al., 1991). In contrast, several proteins can bind Hsp60 but exhibit less dramatic improvements in folding efficiency and a reduced dependence on Hsp10. These include lactate dehydrogenase (Badcoe et al., 1991), dihydrofolate reductase (Viitanen et al., 1991; Martin et al., 1991), α-glucosidase (Holl-Neugebauer et al., 1991), an antibody fragment (Schmidt and Buchner, 1992), and β-lactamase. A list of the tailspike mutants studied by Van Dyk et al. (1989) was 9Tsf(Gly304Val), 9Tsf(Glu344Lys), 9Tsf(Lys382Glu), 9Tsf(Val244Arg), 9Tsf(Glu353Glu), 9Tsf(N39), 9Tsf(U40), 9Tsf(Am232) and 9Tsf(Gly323Asp). In these nomenclature used by Van Dyk et al., these were referred to as 9Ts/13, 9Ts/16, 9Ts/134, 9Ts/304, 9Ts/239, 9Ts/39, 9Ts/40, 9Ts/73, 9Ts/304, and 9Ts/302, respectively.

### Table III

| Phage strain | Cells used for propagation | Incubation conditions | 39°C with Mg²⁺ | 39°C without Mg²⁺ |
|--------------|---------------------------|----------------------|----------------|-----------------|
| Wild-type    | Control                   | 0.8                  | 0.6            |                 |
| Wild-type    | pOF39                     | 0.7                  | 0.7            |                 |
| 5Tsf(Gly403Asp) | Control               | 0.5                  | 2.7e-6         |                 |
| 5Tsf(Gly403Asp) | pOF39                 | 0.4                  | 6.7e-6         |                 |
| 5Tsf(Trp48Gln) | Control                   | 0.6                  | 1.4e-6         |                 |
| 5Tsf(Trp48Gln) | pOF39                     | 0.8                  | 1.2e-6         |                 |
| 5Tsf(Thr294Ile) | Control                 | 1.0                  | 0.8            |                 |
| 5Tsf(Thr294Ile) | pOF39                 | 0.9                  | 0.9            |                 |

1880; Haase-Pettingell and King, 1988). Apparently, GroEL/ES cannot rescue folding defects in all proteins. This reveals an aspect of the selectivity of chaperonin function. Our results suggest that the ability of GroEL to mediate folding is protein-specific rather than allele-specific.
Chaperones and Phage Assembly—The GroE genes were originally discovered as a result of their requirement for bacteriophage morphogenesis. The GroE proteins are found in most prokaryotes, and the GroE complex plays a role in the assembly of the icosahedral capsids of bacteriophage morphogenesis. The GroE complex is composed of the GroEL and GroES chaperones, which are involved in the folding of nascent polypeptide chains and the assembly of viral capsids. The GroE complex is known to disaggregate denatured proteins, refold them, and assist in the assembly of viral capsids. This suggests that GroEL may link coat protein folding to shell assembly. The GroE complex is also involved in the heat-shock response in bacteria, where it can induce the heat-shock response in P22 folding mutants appeared to induce a similar response in Salmonella typhimurium. The GroE complex is known to stabilize an assembly-competent conformation of the viral capsid and is required for the assembly of the icosahedral shell. The GroE complex is also involved in the assembly of the icosahedral capsids of bacteriophage morphogenesis.
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