Substrate Mutations That Bypass a Specific Cpn10 Chaperonin Requirement for Protein Folding*

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The bacteriophage T4 GroES homologue, gp31, in conjunction with the Escherichia coli chaperonin GroEL, is both necessary and sufficient to fold the T4 major capsid protein, gp23, to a state competent for capsid assembly as shown by in vitro expression studies. GroES is unable to function in this role as a productive co-chaperonin. The sequencing and characterization of mutations within gp23 that confer GroEL and gp31 chaperonin-independent folding of the mutant protein suggest that the chaperonin requirements are due to specific sequence determinants or structures in critical regions of gp23 that behave in an additive fashion to confer a chaperonin bypass phenotype. Conservative amino acid substitutions in these critical regions enable gp23 to fold in a GroEL-gp31 chaperonin-independent mode, albeit less efficiently than wild type, both in vivo and in vitro. Although the presence of functional GroEL-gp31 enhances folding of the mutated gp23 in vivo, GroEL-GroES has no such effect. Site-directed mutagenesis experiments suggest that a translational pausing mechanism is not responsible for the bypass mutant phenotype. Polyhead reassembly experiments are also consistent with direct, post-translational effects of the bypass mutations on polypeptide folding. Given our finding that gp31 is not required for the binding of the major capsid protein to GroEL and that active GroES is incapable of folding the gp23 polypeptide chain to native conformation, our results suggest co-chaperonin specificity in the folding of certain substrates.

GroEL and its co-chaperonin, GroES, are stress-inducible proteins, synthesized from the groE operon of Escherichia coli (1, 2). GroEL and GroES are members of the chaperonin or Cpn60/Cpn10 family of proteins and are thought to be general catalysts unrestricted in ability to fold proteins by interacting reversibly with polypeptides to passively prevent or deter incorrect protein folding and aggregation (3, 4). The mechanism of chaperonin-mediated polypeptide folding involves the interaction of GroEL with non-native polypeptide, subsequent GroES association, ATP hydrolysis, and release of the native protein. Both GroEL and GroES are required for E. coli growth at all temperatures, and GroEL has been shown to bind up to 50% of all E. coli proteins in vitro suggesting that the chaperonins have the ability to interact with a wide array of polypeptide substrates (5, 6).

GroEL is initially synthesized as a 60-kDa monomer, which oligomerizes into a decatramer double-donut structure, two stacked rings each with 7-fold symmetry (7, 8). The GroEL double donut structure has the dimensions 14.5 × 16 nm with an inner diameter of 6 nm which enable it to accommodate a 35-kDa protein in the central cavity prior to GroES binding (9). The GroEL-co-chaperonin, GroES, forms a single, seven-membered, domed ring structure consisting of identical 10-kDa subunits (10–13). Mutational analysis of the GroEL apical domain, which faces the opening of the central cavity in the GroEL toroid, indicates an overlap in polypeptide GroES-binding sites (14), and cryoelectron microscopy structural studies show major conformational changes associated with the addition of Mg-ATP and GroES that effectively increase the size of the GroEL folding chamber to accommodate the folding of proteins approximately 60 kDa in size (12, 15). These experiments suggest a critical role for GroES as an intricate player in the binding and release of the polypeptide substrate. However, in vitro experiments demonstrate GroEL-mediated folding of some proteins in the absence of the co-chaperonin (16–18), thus in vivo assessment of the co-chaperonin requirement for folding a particular substrate might be misleading as compared with in vivo determination. In fact, the significance of the co-chaperonin in GroEL-mediated protein folding remains to be fully understood.

In addition to their role in the folding of E. coli proteins, GroEL and GroES are important components in phage assembly pathways. Unlike bacteriophages lambda (1), T5 (19), Mu (20), HK97 (21), and PRD1 (22) which require both GroEL and GroES for growth, bacteriophage T4 is not affected by any of the known missense mutations in the gene encoding the E. coli GroES co-chaperonin. During bacteriophage infection the major capsid protein is synthesized as 56-kDa monomers that form a lattice structure shown to be composed of hexameric arrays of gp23. Before 1970, it was determined that bacteriophage T4 synthesizes a bacteriophage protein that is required to assemble its major capsid protein, gp23, into head structures; without this factor what would now be called inclusion bodies of the major capsid protein accumulate in vivo precluding the formation of proheads (23). This factor, identified as gp31, participates together with the E. coli Cpn60 protein, GroEL, in the correct folding of gp23 (24–28). Several lines of evidence support the interaction of GroEL, gp31, and the T4 major capsid protein during head assembly. A gp23-gp31 interaction is suggested by T4bypass31 phage, which contain mutations in the gene encoding the major capsid protein that appear to obviate the gp31 requirement (29). Specific missense mutations in genes 31 and groEL block T4 propagation at the same point in the head morphogenesis pathway; no head structures

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are assembled and the immature, uncleaved form of gp23 is found to aggregate. Also, certain missense mutations in gene 31 are able to suppress groEL mutations that normally block T4 capsid assembly in an allele-specific manner. Thus, during T4 infection, gp31 appears to replace GroES as the co-chaperonin for GroEL (30, 31).

The nature of the interaction between chaperonin and target protein remains to be determined. The literature suggests this interaction is both indiscriminate and sequence-independent (4). The unique adaptation of the E. coli chaperonin system by bacteriophage T4 might provide an alternative route to study the role of the co-chaperonin in chaperonin-mediated protein folding. However, the specificity requirement for T4 gp31 in conjunction with GroEL to fold gp23 has not in fact been established. T4 infection inactivates E. coli RecA and Rec BCD and replaces these proteins with others of apparently equivalent catalytic properties, gpwvX and gp46/47. Other E. coli enzymes (e.g. DNA polymerase, DNA ligase, valyl-tRNA synthetase) are bypassed or altered for phage enzymes of comparable catalytic properties, sometimes for unknown reasons, whereas phage enzymes of novel specificity (e.g. RNA ligase) are also synthesized (59). We thus proposed to determine by in vivo expression studies the basis for the phage T4 gp31 requirement as follows: whether this is a consequence of T4-imposed changes to the infected cell (e.g. GroES inactivation during infection, limiting functional groES due to host protein synthesis shut-off, increased folding requirements during viral assembly requiring enhanced catalysis, etc.) or whether there is indeed specificity in the GroEL-gp31-meditated folding of the substrate T4 major capsid protein that requires a special factor despite evidence that the E. coli Cpm60-Cnp10 system is capable of generic protein folding.

In this paper, we use expression vectors to show that co-expression of gene 31 with gene 23, in the presence of wild-type GroEL, is necessary and sufficient to form polyhead structures in vivo and that GroES is apparently unable to function in this role. Polyheads, which are open tubes formed exclusively of gp23, require proper folding and oligomerization of the major capsid protein and are thought to display the same structure found in active T4 proheads (Fig. 2E; cf. Ref. 32); polyhead formation is thus indicative of folded gp23. Although folding and polymerization of the wild-type major capsid protein strictly require GroEL and gp31, we show that polyheads are produced in the absence of both proteins by T4 phage containing two types of known bypass31 mutations in the major capsid protein, both in vivo and in vitro. Characterization and analysis of T4bypass31-2 mutant phage, which are able to grow in the absence of chaperonins at high temperature (>40 °C), identify three missense mutations that confer chaperonin-independent folding to the mutant gp23 protein in an additive fashion. These data suggest that the ability of the gp23N protein to fold independently of chaperonin assistance is tightly associated to certain localized sequence determinants within the major capsid protein. Mutagenesis of one of these sites allows us to investigate the specificity of amino acid replacements that confer the phenotype, determine its chaperonin independence, and investigate a proposed mechanism of translational pausing (33, 34) which might promote protein folding in the absence of chaperone assistance.

EXPERIMENTAL PROCEDURES

Materials—Molecular biology reagents were purchased from either Boehringer Mannheim, New England Biolabs, or Life Technologies, Inc. Antibiotics against GroEL and GroES were purchased from Sigma; antisera against T4 gp31 and gp23 were described previously (24, 32). Tran35S-label was purchased from NEN Life Science Products.

Bacterial and Phage Strains—The bacteria and phage strains used are listed in Table I. E. coli B40 su1,1, B40 su1,1, CR63, LE392, B8 supr and P301 sup, were used as permissive and non-permissive hosts for T4 amber mutants. Other suppressor strains, which were kindly provided by J. Miller (see Ref. 35), were used in the analysis of the bypass31-2 site presented in this work. pTrc99A (Amersham Pharmacia Biotech) and pET-derived plasmids (Novagen) were used to transform host strains for mutant and expression in HMS174(DE3), BL21(DE3), and their non-DE3 ET3 derivatives (Novagen). T4D- was the T4 wild-type strain. A phage mutant is described by its gene number, followed by the type of mutation and the name of the specific mutation. Multiple mutations are denoted the same way with a hyphen between mutations. Other phage strains used in these experiments include the following: λ 21 T421(AMH29) 23 T422TAK31(AMHNS) T431(AMNG71)-31(AMN54)- BYP1, T4bypass31-1-31(AMN54); BYP2, T4bypass31-2-31(AMNG71)-31(AMN54); and the double bypass, BYP1+2, T4bypass31-1-2 mutants were used in complementation experiments. DNA from the double bypass31 strain was used as the DNA source for the construction of the pETBY plasmid.

Plasmid Construction and Site-directed Mutagenesis—Constructs were made by inserting the target T4 gene downstream of the T7 promoter in either the pET3a (Novagen) expression vector using the Ndel and BamHI restriction sites or the pTrc99A (Amersham Pharmacia Biotech) expression vector using the XbaI restriction endonuclease site. PCR primers, synthesized by the Biopolymer Center at the University of Maryland, Baltimore, School of Medicine, complementary to sequences of T7 downstream of GroES, were used to introduce Asel and BamHI restriction enzyme sites and to isolate the gene from T4 or T4bypass31-1-2 mutant DNA. Constructs were made by inserting the target T4 gene into the pET3a (Novagen) expression vector, creating pET23 and pETBY, respectively. Isolating an Asel-BgII fragment containing gene 31 from M13mp11-T5 and directly cloning into the pET3a vector as described above (30) constructed pET31. An XhoI fragment from this vector, containing gene 31 and its Shine-Delgarno sequence, was then directly cloned into pTrc99A (Amersham Pharmacia Biotech), creating pTrc31. The double construct was made by inserting a BgII-EcoRI fragment-containing gene 31 from pET31 into pET23 previously cleaved by BamHI and EcoRI. Although the T7 promoter is used to initiate transcription, the natural Shine-Delgarno site is maintained for each gene. Individual plasmids were transformed into the E. coli groEL44 strain CG2241, which is non-permissive for T4 growth at 37 °C or above and whose target plasmids can be induced by λCE6 and HMS174(DE3), which is a lysogenic strain carrying an IPTG-inducible gene for T7 polymerase (36). The plasmid pEGS1, a generous gift from E. Eisenstein, contains the gene encoding λCE6 and was transformed into the strain JZ483 and used in GroES over-expression studies (37). Transformants were screened by marker rescue experiments, and protein expression was confirmed by 12.5% SDS-PAGE or, in the case of gp31, 16% Tricine-polyacrylamide gel electrophoresis, followed by Western blot analysis (data not shown).

The sequence of gene 23 from both wild-type T4 and T4bypass31-1-2 was determined by automated sequencing at the Biopolymer Laboratory Core Facility, University of Maryland at Baltimore. Site-directed mutagenesis of gene 23 (38) used wild-type and mutagenic primers, synthesized at the University of Maryland Biopolymer Laboratory at University of Maryland, Baltimore, to introduce site-directed missense and amber mutations into wild-type gene 23. The mutated gene 23 was then cloned into the pET3a expression vector as described previously and recombined into bacteriophage T4. The mutations were subsequently confirmed by automated sequencing.

Protein Expression and Isolation of Polyhead Structures—For plasmid expression and polyhead isolation experiments, plasmid-containing bacteria were grown to an A600 of 0.6 in M9S media supplemented with ampicillin (150 µg ml−1) and induced for 4 h at 37 °C with the addition of IPTG to a final concentration of 0.4 mM, for pET-derived plasmids, or of 5 mM, for pTrc31 and pEGS1. The groEL mutant strain CG2241 containing pET23, pET23S1, or pETBY was grown in M9S (39) media supplemented with 0.2% maltose and ampicillin (150 µg ml−1) to an A600 of 0.6 at 37 °C. Expression was induced by λCE6 infection at 37 °C for 4 h (36). For Western blot analysis, samples were electrophoresed in a 12.5% SDS-polyacrylamide gel and electroblotted onto Immobilon-P (Millipore) and probed with antisera. Rabbit antibodies directed against gp23 were used in conjunction with the chemiluminescent ECL technique (Amersham Pharmacia Biotech) for detection of gp23 protein.

1 The abbreviations used are: PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactopyranoside; WT, wild type; m.o.i., multiplicity of infection; PAGE, polyacrylamide gel electrophoresis; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine.
The procedure for polyhead isolation has been previously published (40). HMS174(DE3) bacteria containing either pET2331 or pETBY were grown to an A600 of 0.6. Bacteria were induced for 4 h at 37 °C and centrifuged at 5,000 rpm in a Sorvall SS-34 rotor at room temperature for 10 min. The cell pellets were resuspended in (0.01 original culture volume) 100 mM KPO4, pH 7, 10 μg/ml pancreatic DNase I (Sigma), 5 mM EDTA. Cell number was normalized by A600 readings. All subsequent steps were performed at room temperature. 100 μM lysozyme was added to the bacteria to begin lysis, completed by the addition of 1–2 drops of chloroform, and confirmed visually with the light microscope.

After centrifugation at 5,000 rpm in a Sorvall SS-34 rotor for 20 min at 4 °C, the pellet was repeatedly centrifuged and washed four times in buffer A (100 mM KPO4, pH 7, 10 mM MgSO4), and the supernatants were pooled. The pooled supernatants were centrifuged at the above speed to remove any particulate debris. The clarified supernatant was then centrifuged at high speed (18,000 rpm in a Sorvall SS-34 rotor) for 60 min to pellet the polyhead structures. Repeated low and high speed centrifugations were used to remove cellular debris, including contaminating vesicles, to yield a relatively pure gp23 polyhead sample.

**In Vitro Denaturation and Refolding of the T4 Major Capsid Protein**—Once isolated, polyheads consisting of either wild-type gp23 or gp23 Mutants were dialyzed against buffer B (1 mM KPO4, pH 7, 1 mM MgSO4) at 4 °C. Polyheads are destabilized under these conditions and depolymerize (40). Samples were then centrifuged at 18,000 rpm in an SS34 rotor for 20 min at 4 °C. The purpose of this step was to pellet any intact polyhead structures and to take only the soluble proteins in the supernant for subsequent analysis. The supernant was observed by electron microscopy and was found not to contain any polyhead structures. Half of the sample was used for reassembly and half for denaturation/refolding experiments. Urea was added to the depolymerized gp23 and incubated for 45 min, and the urea was then slowly dialyzed away with buffer B to initiate polypeptide renaturation. Once the urea content reached micromolar concentrations, the dialysis buffer was diluted 1:10 with fresh buffer B. Polyhead reassembly was initiated by dialyzing the sample against 25 mM KPO4, pH 7, 1 mM MgSO4 at room temperature for 1 h. Over a period of 3 h the KPO4 concentration in the dialysis buffer was increased to a final concentration of 100 mM. Samples were then centrifuged at 18,000 rpm in an SS34 rotor for 60 min to pellet the polyhead structures. Repeated low and high speed centrifugations were used to remove cellular debris, including contaminating vesicles, to yield a relatively pure gp23 polyhead sample.

**Electron Microscopy**—Methods for the fixation, dehydration, embedding, and thin sectioning of bacteria are a slight modification of previously published procedures (41). Polyhead-containing samples were preserved in 1% final concentration of glutaraldehyde and negatively stained with 1% uranyl acetate for observation.

**Complementation Experiments**—Bacteriophage growth on the groES
strain, JZ483, and the suppressor minus strain, B', was determined under various conditions by calculating lambda, T4, or site-directed mutant phage burst sizes. The burst size is given as the number of viable phage progeny produced per infected bacterium. JZ483 bacteria containing either pEGS1 or pTrc31 plasmids were induced by the addition of 4 mM IPTG at an A600 of 0.4. JZ483 bacteria, the infected with either lambda or T43f (AmNG71) phage at an A600 of 0.1. The phage were allowed to adsorb to the bacteria for 7 min after which antibody directed against either lambda or T4 phage was added. Bacteria were incubated and incubated for 90 min followed by the addition of chloroform to release progeny phage. T4 phage was then washed on the surface and the suppressor minus strain, B', and the suppressor plus strain CR63 and lambda phage were titrated on LE392 to determine the phage yield per infected cell. 

Immunoprecipitation and Radiolabeling Experiment—E. coli B' were grown at 37 °C in M9 medium (39) to an A600 of 0.3 which corresponds to 2 x 10^6 bacteria/ml and infected with one of the following phage: T43f (AmB2), T4', T43f (AmNG713131AmNG54), or T4bypass31(1+2)-31/AmNG543131AmNG71) at an A600 of 5, or with no phage. Bacteria were superinfected with the same A600 at 8 min. TrnasS-sytoe-methine label (NEN Life Science Products) was added to the media at 10 μCi/ml just prior to infection to label GroEL protein in the uninfected bacteria and at 10 min post-infection to label late T4 structural proteins in T4-infected bacteria. After 15 min post-infection, 50% of the gp31 synthesis ceases by 15 min (24), bacteria were placed in an ice-water bath, and excess non-radioactive methionine and cysteine were added together with a casen amino acid (CAA) mixture (0.2 ml 20% CAA/ml infected cell culture). Infected bacteria were then pelleted in a Sorvall centrifuge (5,000 rpm in a SS-34 rotor for 8 min at 4 °C). Cell pellets were then stored in a −80 °C freezer until use or directly used for immunoprecipitation with anti-GroEL or anti-GroES antibody. The following procedure is based on a 2-ml original culture volume. 0.4 ml of lysis buffer containing 1 mM ATP was added to the cell pellets. Lysis buffer contained 20 mM Tris-Cl, pH 7.5, 80 mM KCl, 5 mM MgCl2, 1% Triton X-100, 1% Nonidet P-40, and 1% bovine serum albumin. The bacteria were incubated for 37 °C for 1 h to allow for lysis to occur. The samples were centrifuged to remove the bacterial debris (16,000 rpm in a microcentrifuge or 5,000 rpm in a Sorvall SS34 rotor at room temperature). All subsequent steps were performed at room temperature unless otherwise indicated, and 1 mM neutralized ATP was added along with each manipulation of the cell lysate. A 1:100 dilution of normal rabbit serum was added to the supernatant, and the samples were incubated for 1 h on an orbital shaker. 0.02 ml of a 50% protein A-Sepharose slurry (Amersham Pharmacia Biotech) was then added to the supernatant for 1.5 h. The protein A-Sepharose was gently pelleted at 1,000 rpm in a microcentrifuge or by gravity, and the pre-cleared supernatant was used in subsequent steps. 0.5 ml of dilution buffer (20 mM Tris-Cl, pH 7.5, 5 mM MgCl2, 1% Triton X-100, 1% Nonidet P-40, and 1% bovine serum albumin) was added to dilute the KCl and detergent concentrations that might destabilize the tertiary (Cpn60-Cpn10-polypeptide) complex. The lysates were split into two groups as follows: one which was not treated with anti-GroEL or anti-GroES antibody, and one which was treated with a 1:1000 dilution of GroEL antibody. Similarly, precipitation of GroES was confirmed by Western blot analysis with anti-GroEL or anti-GroES antibody. Anti-GroEL or anti-GroES antibody. Polyheads isolated by differential centrifugation of cell lysates (40) were analyzed by SDS-PAGE and Western blot analysis (Fig. 1, B and C). Lanes 2 and 3 of Fig. 1, B and C, show that the formation of gp23 polyhead structures in a wild-type host (i.e. bacteria containing normal levels of GroEL and GroES) requires the co-expression of gene 31. In contrast, polyheads are formed by the mutant gp23, gp23wt, in a wild-type host, even in the absence of gene 31 co-expression (Fig. 1, B and C, lane 4). When pET23, pET2331, and pETBY plasmids were induced to express their target genes in the GroEL-deficient strain, CG2241, at the non-permissive temperature, only gp23wt expressed from the pETBY plasmid was able to form polyhead structures in vivo (Fig. 1, B and C, lanes 5–7). Gene expression from all three of the plasmids was far lower in the groEL44 strain CG2241 at 37 °C, probably both because of the ACE6 induction and the GroEL deficiency of the mutant host at a temperature semi-permissive for growth. Comparable amounts and stabilities of gp23 resulted from all three constructs (Fig. 1A, lanes 5–7, and Western blotting, data not shown); however, only expression of gene 23 was shown to yield polyheads both in the absence of gp31 and in the groEL44-deficient host, as judged by SDS-PAGE, Western blotting (Fig. 1, B and C, lanes 5–7), and by electron microscopy (Fig. 2, D and E). Thus, production of polyheads is not closely related to the amount of gp23 accumulation since polyheads can be formed when expression levels are low as well as high (Fig. 1, A and C, cf. lanes 7 and 3).

Aggregation analysis (data not shown) (23, 42) and electron microscopic observations of the induced plasmid-containing bacteria were in complete agreement with the conclusions drawn from PAGE. Electron micrographs of thin sectioned bacteria expressing gene 23wt from pET2331, pET23, or gene 23 from pETBY in groEL + and groEL − hosts are shown in Fig. 2, A–D. A comparison of Fig. 2, A and B, shows multilayered polyhead tubules are abundant in bacteria-expressing gene 23wt in the presence of functional GroEL and the T4 co-chaperonin gp31 (an average of 44 polyheads/section). Fig. 2A shows both a longitudinal view of polyheads in the lower cell, and a cross-sectional view of polyheads formed as bundles of centric rings, indicative of multilayered polyheads, in the upper cell. The latter structures are expected to accumulate because inner gp23 layers provide an assembly template for unassembled major capsid protein in the absence of accessory T4 prohead core proteins (60). As seen in Fig. 2B, bacteria expressing only gene 23wt were never observed to produce polyheads.
but rather form large inclusion bodies presumably containing aggregated 23 protein. Multi-layered polyheads were also found in bacteria expressing gene 23BY even in the absence of functional gp31 and GroEL proteins (Fig. 2, C and D). Fig. 2E shows an electron micrograph of a negatively stained polyhead isolated from CG2241 bacteria expressing pETBY (expresses mutant gene 23 bypass, gp23BY). A–D, thin sections of pET331/HMS174 (DE3) (A); pET23/HMS174 (DE3) (B); pETBY/HMS174 (DE3) (C); and pETBY/G2241groEL44 (D). E, negatively stained polyhead isolated from CG2241groEL44 bacteria expressing pETBY at the non-permissive temperature. F, negatively stained gp23BY multilayered polyhead reassembled after in vitro renaturation from 6 M urea in the absence of chaperone proteins. Polyhead tubules and cross-sections of tubes are shown in A, C, and D. Only inclusion bodies can be seen in HMS174 (DE3) bacteria expressing the wild-type gp23 protein in the absence of gp31 (B) whereas both polyheads and inclusion bodies are observed in bacteria producing the gp23BY protein (C and D). Magnification of electron micrographs in A–D, × 50,000. Magnification of E and F, × 200,000.

FIG. 2. Polyhead formation in vivo following expression vector synthesis of the major capsid protein gp23, but not of the gp23BY mutant capsid protein, requires synthesis of the co-chaperonin gp31 and functional GroEL chaperonin as determined by electron microscopy of thin sectioned E. coli. HMS174 (DE3) and the mutant groEL44 strain CG2241 contained expression vectors pET23 (expresses gene 23), pET231 (expresses genes 23 and 31), and pETBY (expresses mutant gene 23 chaperonin bypass). Coomassie Blue-stained SDS-PAGE of cell lysates before (A) and after (B) polyhead isolation (cf. “Experimental Procedures”); C, Western blot of B with anti-gp23 antibody. Lanes 2–4 are 2/3 the concentration of lanes 5–7. Molecular mass markers are indicated in kDa. Lane 1, partially purified gp23 control (gp23 arrow) is 56 kDa; lane 2, pET23/HMS174 (DE3); lane 3, pET2331/HMS174 (DE3); lane 4, pETBY/HMS174 (DE3); lane 5, pET23/G2241groEL44; lane 6, pET2331/G2241groEL44; and lane 7, pETBY/G2241groEL44.

The abundance of polyheads was markedly lower in HMS174 (DE3) bacteria expressing gene 23BY when compared with bacteria co-expressing genes 23WT and 31. Also, bacteria expressing gene 23BY appear as an intermediate between bacteria expressing gene 23WT with and without the T4 co-chaperonin gp31; they contain polyheads as well as inclusion bodies. This suggests that folding and/or polymerization of gp23, synthesized from the bypass311(1+2) mutant DNA, is less efficient than that of the wild-type protein, and this is consistent with the proportionate decrease in the T4bypass31 phage yield when compared with T4 wild type (Table IV (29)).

The T4bypass31 mutant phage appear to have simultaneously lost the gp31 requirement and to have gained the ability to grow on the E. coli groEL44 mutant CG2241 (29, 43). Table II shows a more extensive analysis of T4bypass31 phage growth on several groEL and groES mutant strains. As determined previously, all of the groEL and groES mutations affect
lambda phage growth viability, whereas wild-type T4 phage growth is only affected by some mutations in the groES gene (Table II) (1, 30, 31). The T4 phage containing a double amber mutation in gene 31 and wild-type gene 23 is unable to grow in any of the mutant strains because of the lack of gp31, confirming the T4 co-chaperonin specificity requirement for gp31. Unlike wild-type T4, the T4 by bypass31 single and double mutants are unaffected by any of the groEL or groES mutations tested. This suggests the T4 by bypass31 single and T4 by bypass31-2 mutations each confer changes in the structure of the major capsid protein which allow it to fold independently of GroEL, GroES, and gp31. In vitro refolding studies using purified proteins also support our in vivo conclusion that the bypass31 mutations confer chaperonin-independent folding of the major capsid protein (cf. “Experimental Procedures”). As expected, both wild-type gp23 and gp23BY proteins form polyheads from a depolymerized state (40, 42). However, gp23BY appeared to more readily dissociate from polyheads in low ionic strength, low temperature buffers (42). Although the yield was low, multilayered polyhead structures, previously observed exclusively and intracellularly in vivo, were observed upon renaturation of the purified gp23BY, but not the wild-type gp23 protein, from 6 m urea in the absence of chaperonins (Fig. 2F).

Functional Differences of Cpn10 Homologues GroES and gp31—While our work was in progress, it was shown that gp31 can act as a functional surrogate for GroES and as a general co-chaperonin during the reassembly of ribulose-bisphosphate carboxylase/oxygenase (Rubisco) protein and during lambda and T5 infection (19). Thus it is a distinct possibility that GroES and gp31 are functionally or catalytically interchangeable and that gp31 is produced because available GroES protein is inactivated or limiting during T4 infection (cf. Introduction). If gp31 and GroES can both interact with GroEL as well as with the same substrates with comparable efficiency, one might expect the overproduction of GroES protein to compensate for a defective 31 protein during T4 infection. Complementation testing was used to determine whether gp31 and GroES as over-produced from pTrc31 and pEGS1, respectively, are functionally interchangeable in vivo. Both pTrc31 and pEGS1 were transformed into the groES42 mutant JZ483 and induced with IPTG to express their target genes. The bacteria were then infected with lambda or T4 by NG71 phage, and the burst size for each phage was determined. Fig. 3A shows lambda phage growth rescued by the over-expression of groES from pEGS1, approximately 92% of lambda phage growth on a fully permissive strain. In agreement with previous findings (19), over-expression of gene 31 can also rescue lambda phage growth although less effectively than GroES, approximately 8% of control. In the case of T4 phage growth, however, over-expression of groES does not rescue T431-(AmNG71) phage growth, approximately 0.002% of control (Fig. 3B). Burst size measurements were also carried out in another groES mutant, groES619, with similar results, and both expression vectors produced the cloned gp31 and GroES co-chaperonins at very high levels following induction (42). These data demonstrate that GroES and gp31 are not equally interchangeable. Although gp31 can at least partially compensate for a defective GroES, it does not appear to rescue lambda phage growth as effectively as GroES. Furthermore, overproduction of GroES does not compensate for a gp31 deficiency, demonstrating that T4 specifically requires gp31 for the proper folding and oligomerization of its major capsid protein, gp23, consistent with the in vivo expression studies.

Interaction of Wild Type and bypass31 Mutant Major Capsid Protein with GroEL—Co-immunoprecipitation experiments using anti-GroEL polyclonal antibody were designed to determine whether the wild-type gp23 and the mutant gp23BY both interact with GroEL during T4 infection and to determine whether gp31 is involved in the initial binding of the gp23 polypeptide to GroEL. E. coli BY2 bacteria were grown to an A600 of 0.3 in labeling media and either left uninfected or infected with one of the following phage at a m.o.i. of: 5: T421 (AmH29), a head assembly proteinase-deficient strain; wild-type T4; T431-(AmNG71)-31 (AmN54); or T4 by bypass31-1-2 (AmNG71)-31-31. Bacteria were labeled with Tran35S-label at 10 min post-infection for 5 min to specifically radiolabel T4 structural proteins. Fig. 4A (lanes 1–5) shows abundant T4 late proteins labeled in whole cell extracts including gp23 and gp23*, which is the N-terminal processed form of the major capsid protein found in the mature prohead structure. Immunoprecipitation of cell lysates with GroEL antibody identified the major capsid protein as a T4 late protein that interacts with the Cpn60 (Fig. 4A, lanes 6–10). Co-immunoprecipitation experiments done in parallel without the addition of GroEL antibody showed some nonspecific precipitation; however, the amount of nonspecific intact gp23, wild-type or mutant, was negligible (Fig. 4A, lanes 11–15). At host protein synthesis ceases upon infection, bacteria were labeled prior to infection to show precipitation of endogenous GroES together with some E. coli proteins possibly associated with GroEL (Fig. 4A, lane 10). Fig. 4A (lanes 7 and 9) shows that both the wild-type gp23 and the mutant gp23BY co-immunoprecipitate with GroEL from the infected cell lysates. Thus, the bypass mutations do not prevent binding of gp23BY to GroEL, as expected from the better folding of gp23BY in the presence of GroEL (42). Although this gp23BY-GroEL interaction is not necessary for functional folding of the mutant major capsid protein, the presence of functional GroES and gp31, but not GroES, enhances gp23BY folding (42). The co-immunoprecipitation of wild-type gp23 with GroEL even in the absence of gp31 (Fig. 4A, lane 8) suggests that gp31 is not necessary for binding to GroEL but that it is required for functional protein folding. Immunoblotting of the cell extracts and co-immunoprecipitates with GroES and gp31 antisera shows that both GroES and gp31 are present and are found to interact with GroEL during T4 infection (Fig. 4B, lanes 6–15, and data not shown).

Immunoprecipitation experiments using extracts prepared following induction of uninfected bacteria containing the PET23, pET22331, and pETBY expression vectors yielded comparable results (data not shown), i.e. gp23, and gp23BY were associated equally with GroEL in the presence or absence of gp31, and GroES continued to be associated with GroEL following induction of gp31 synthesis.

Our work clearly demonstrates the specificity of the gp31
requirement for the folding of the T4 major capsid protein, gp23. As GroEL and GroES intracellular levels are comparable, it is interesting to speculate as to what occurs in vivo during T4 infection when both GroES and gp31 are present. Electron micrographs of negatively stained purified gp31 show a ringed structure with a central hole that is similar in dimension to GroES (42). Assuming that gp31 forms a 7-mer oligomeric structure, the number of gp31 molecules synthesized during T4 infection can be calculated from immunoquantitation measurements (24) to be approximately 7,800 gp31 septemers/cell. Intracellular concentrations of GroEL and GroES have been determined previously to be approximately 1,600 GroEL 14-mers/cell and 3,000 GroES 7-mers/cell (44). Thus, it is possible that this difference in co-chaperonin availability in the infected host increases the likelihood that gp31 will interact with GroEL and thus, favors gp23 folding.

**Identification and Characterization of Mutations in the T4 Major Capsid Protein That Confer Chaperonin-independent Protein Folding.**—The pETBY plasmid was used to sequence the mutations in gene 23 that confer chaperonin-independent folding to the major capsid protein. The T4bypass31-1 mutation is a temperature-sensitive, conservative single amino acid replacement located near the carboxyl terminus of gp23 and converts alanine 455 to valine 455 in agreement with UV mapping data (43). The T4bypass31-2 mutation, which resisted genetic mapping (43), was found to consist of three single base mutations in the middle of gene 23 which convert glycine 292 to serine 292, valine 306 to isoleucine 306, and valine 307 to isoleucine 307. The effect of each of these amino acid changes in conferring the bypass mutant phenotype could be determined by introducing the changes singly and in combination into wild-type gene 23 followed by scoring for growth on groEL mutant E. coli strains and in the absence of functional T4 gene 31.

Overlap PCR mutagenesis was used to introduce site-directed mutations into genomic wild-type gene 23 to determine whether all three mutations at the bypass31-2 site are required for the bypass phenotype. The various mutated gene 23 sequences were cloned into the pET3a vector and subsequently recombined into bacteriophage T431(NG71)31(NG54), which does not produce intact gp31 in a suppressor minus host bacterium. By using this technique several combinations of the three mutations were tested for the ability to grow on groEL and groES mutant bacterial strains at a temperature that was non-permissive for the groE mutations (Table III). As expected, the wild-type gene 23-containing phage are unable to grow in the absence of either gp31 or GroEL, and the T4bypass31(1+2) phage, also containing double amber mutations in gene 31, grow well on all strains tested. All of the site-directed mutant phage recombinants grow on CR63, the fully permissive strain. The inability of T4316VV, T415S-I1, and T415S-I2 to grow on the suppressor-negative B6 strain demonstrates the inability of the single site mutated phage to bypass the gp31 requirement. Analysis of phage growth on the groE mutant strains, which are also suppressor-negative, shows that the two isoleucine mutations are essential to conferring chaperonin-independent folding to the gp23 polypeptide. Although the presence of the mutant serine at position 292 is not essential for the bypass phenotype, the plaque size of T4156GI2 was significantly adsorb to the bacteria for 7 min after which antibody directed against either λ or T4 phage was added. Infected bacteria were then diluted to 10⁻¹ and plated immediately to determine number of infected bacteria. The remainder of the bacteria was incubated 90 min at 37 °C, lysed with chloroform, and titered to determine the burst sizes (the number of viable phage progeny produced per infected bacterium). Burst measurements reported for λ are averaged from six independent experiments, for T431 (NG71) from seven independent experiments.
smaller than that of the serine containing T4136SB (42), suggesting that serine 292 does enhance the ability of these phage to grow in the absence of functional GroEL or gp31. As demonstrated by phage growth experiments, the additive contribution of all four bypass mutations (bypass31-1 [V] and bypass31-1 [S-II]) is required for optimal gp23 folding in the absence of gp31 and GroEL. Analysis of the three mutations found in the T4 bypass31-2 mutant phage indicates that these mutations also behave in an additive fashion, the contribution of the Gly—Ser change most apparent when accompanied by the two isoleucine mutations (Table III). The results of burst size determination of site-directed T4 mutant phage infections of the suppressor minus strain B° provide another measure of the ability of these phage to grow in the absence of gp31 and is in complete agreement with the conclusions drawn from plaque formation and morphology (Table IV). In summary, characterization of the three mutations at the bypass31-2 site show that all three mutations (S-II) contribute to optimal growth in the absence of GroEL and gp31 and that the two isoleucines are essential for chaperonin-independent protein folding.

**Fig. 4.** gp23 and GroES interact with GroEL following T4 infection in the presence or absence of gp31 co-chaperonin synthesis. Immunoprecipitation of the major capsid protein gp23 in T4 mutant infected E. coli B° bacteria with GroEL antibody (A) or GroES antibody (B). A shows autoradiograms of 8% SDS-PAGE of 35S-amino-acid-labeled cell lysates (WCX) and cell lysates immunoprecipitated with GroEL antibody (αGroEL) or without antibody (−ab). Lanes indicate infection of B° bacteria as follows: lane a, T423 (AmN54); lanes 1, 6, and 11, T423 (AMB2; lanes 2, 7, and 12, wild-type T4; lanes 3, 8, and 13, T431 (AmNG71)-31(AmN54); lanes 4, 9, and 14, T4bypass31 (1+2)-31(AmNG71)-31(AmN54); lanes 5, 10, and 15, uninfected bacteria. The positions of GroEL (65 kDa) and gp23 (56 kDa) are indicated. B, 17% SDS-PAGE of cell lysates that were immunoprecipitated with GroES antisera and immunoblotted with either GroES antisemur or GroEL antisemur, as indicated. Lane 1, purified gp31; lane 2, purified GroES-GroEL mixture; lane 3, T423 (AMB2); lane 4, wild-type T4; lane 5, T431 (AmNG71)-31(AmN54); lane 6, T4bypass31 (1+2)-31(AmNG71)-31(AmN54); lane 7, uninfected bacteria; lane 8, uninfected bacteria run in parallel without GroES antisemur addition during the immunoprecipitation.

**Examination of a Translational Pausing Mechanism for Chaperonin-independent Folding**—The bypass31-2 mutations are of particular interest because they confer chaperonin-independent folding even at high temperatures where chaperonins are especially required for most proteins. A curious observation is that all of the bypass mutations (the single bypass31-1 and multiple bypass31-2) are missense mutations that cause a reiteration of a preceding amino acid (Ser-Ser, Ile-Ile, and Val-Val). The introduction of an infrequently used T4 codon by the missense mutation that introduces a serine at position 292, AGU, not found in gene 23 and rarely in T4 structural genes (45), suggested a role for translational pausing in the chaperonin-independent folding of the bypass31-2 mutant major capsid protein due to low charged tRNA abundance. Such a mechanism has been proposed to account for codon usage differences within protein domains and interdomain regions (34, 46), and lowered translation has, in fact, been shown to account for enhanced folding of a yeast protein (33). In addition, some gene 31 mutations suppress an E. coli rho gene mutant defective growth phenotype by an unknown mechanism which could be interpreted to reflect coupling of protein folding to concurrent transcription-translation (27, 47).

The introduction of an amber codon at position 292 of the wild-type major capsid protein enabled translational pausing to be examined as a potential mechanism in mediating chaperonin-independent protein folding, since under suppression conditions amber codons should be translated with a significant delay as compared with other codons (48). This approach simultaneously allowed the influence of the amino acid substitution at the am position to be assessed by using tRNA suppressors to incorporate amino acids of various sizes and chemical attributes. The TAG codon was introduced either as the sole mutational change (AmB2) or with the addition of the two isoleucine residues at positions 306 and 307 (AmB2-II). These amber-containing genes (AmB2 and AmB2-II) were recombined into an otherwise wild-type T4 phage, resulting in T423(AmB2), or into a phage containing a temperature-sensitive mutation in gene 31, resulting in T423(AmB2)31(tsA70) and T423(AmB2-II)31(tsA70). Fig. 5 summarizes the effects of amino acid substitutions at position 292 of the major capsid protein tested by infecting various tRNA suppressor strains with the recombinant phage at 30 and 41 °C. Analysis shows that the bulky aromatic amino acids phenylalanine and tyrosine are detrimental to the folding of the gp23 polypeptide chain and that all other amino acids substitutions tested allowed for growth of the T423(AmB2) phage. Tests with T423(AmB2)31(tsA70) are similar to that described above at the permissive temperature and demonstrate that none of the amino acid substitutions is able to confer a bypass phenotype in the absence of the isoleucine residues at positions 306 and 307.
Growth of T4\(^23\)(Am\(^B2\)-II-31\(^{tsA70}\)) at the non-permissive temperature shows a wide variety of amino acid substitutions (including charged, uncharged, and small apolar substitutions) allow for growth in the absence of gp31 when present in conjunction to the two isoleucine residues. An interesting and initially surprising finding was that phage which incorporate a leucine at position 292 grow normally at 30 and 41 °C in the presence of wild-type gp31 and when the temperature-sensitive gp31 protein is present and functional at 30 °C. However, when tested at the non-permissive temperature, leucine has an apparent dominant negative effect on the isoleucine-induced bypass phenotype, reconverting the polypeptide to a chaperonin-dependent state. These data do not support a translational pausing mechanism for the chaperonin-independent folding phenotype but rather indicate that the amino acid substitutions themselves are critical post-translationally and that this region of the polypeptide is critical for the specific chaperonin-mediated folding requirement. This again demonstrates the importance of the specific amino acid sequence in this particular region of the major capsid protein on polypeptide folding and on chaperonin dependence; however, the specific differences are difficult to interpret since subtle, conservative amino acid replacements yield a mutant phenotype.

### Table IV

| Bacteriophage |
|---------------|
| T4\(^+\) | Gly   | val  |
| 31\(-\) | Ser   | ile   |
| BY(1+2) 31\(-\) | Ser | ile   |
| 156SB | Ser   | ile   |
| 136SW | Ser   | ile   |
| 136SB | Ser   | ile   |
| 156G12 | Gly   | ile   |
| 15S-11 | Ser   | ile   |
| 15S-12 | Ser   | ile   |

Bacteriophage strain | Relevant gene 23 sequence | Burst size | S.E. |
--- | --- | --- | --- |
T4\(^+\) | G----V V----A- | 77.5 | 6.5 |
T4(31\(^{AmNG71}\))-(31\(^{AmN54}\)) | G----V V----A- | <0.01 | 0.004 |
T4 bypass31\(-2\), 31\(-\) | S----I I----V- | 10.6 | 2.6 |
T4 156SB | S----I I----V- | 33.0 | 4.0 |
T4 156G12 | S----I I----V- | 1.0 | 0.21 |

The burst size of T4 phage containing the indicated mutations in gene 23, introduced by site-directed mutagenesis, was determined on the suppressor minus strain B\(^-\). With the exception of wild type, all phage used in this study contain both T431\(^{AmNG71}\)-31\(^{AmN54}\) mutations. Bacteria were grown to an A\(_{600}\) of 0.3 and infected with phage at an m.o.i. of 0.1. The phage were allowed to adsorb to the bacteria for 3 min, after which T4 antiserum was added to inactivate unadsorbed phage. Bacteria were diluted to 10\(^{-4}\) and incubated at 37 °C for 90 min. T4 phage was titered on B\(\text{e}^{+}\) and CR63 (sup\(D\)). The results of three independent experiments were averaged and S.E. is indicated. The burst size is given as the number of viable phage progeny produced per infected bacterium. pfu, plaque-forming units.

### Table III

Characterization of T4 phage modified by site-directed mutagenesis on groEL- and groES-defective strains

| CR63 (sup\(B\)) | Be (sup\(D\)) | CG2241 | CG2246 | CG2244 | JZ483 |
|---|---|---|---|---|---|
| T4\(^+\) | ++ | ++ | - | - | ++ | ++ |
| 31\(-\) | + | - | - | - | - | - |
| BY(1+2) 31\(-\) | ++ | ++ | ++ | ++ | ++ | ++ |
| 156SB | ++ | ++ | ++ | ++ | ++ | ++ |
| 136SW | ++ | ++ | ++ | ++ | ++ | ++ |
| 136SB | ++ | ++ | ++ | ++ | ++ | ++ |
| 156G12 | ++ | ++ | ++ | ++ | ++ | ++ |
| 15S-11 | ++ | ++ | ++ | ++ | ++ | ++ |
| 15S-12 | ++ | ++ | ++ | ++ | ++ | ++ |

Wild-type gene 23 was modified by site-directed mutagenesis and recombined into bacteriophage T4 to create phage with various combinations of the three mutations identified at the bypass31\(-2\) site. Serial dilutions of wild-type T4 and T4 phage containing the mutations indicated above (in addition to 31\(^{AmNG71}\)-31\(^{AmN54}\)) were spotted onto lawns of sup\(B\), sup\(D\), CR63, CG2241, CG2246, CG2244, and JZ483. Phage growth is indicated as follows: ++, very good growth; +, moderate growth with normal plaque size; +/-, poor phage growth with small plaque size (<1 mm); and --, no phage growth.
Our work shows that a bacteriophage T4 GroES homologue is required to fold the major capsid protein because of specific structure(s) in the gp23 molecule. Despite these GroES-refractory structures, mutations in the gp23 polypeptide can confer chaperonin-independent folding. This interpretation of the by-pass mutants makes the possibility that the T4 major capsid protein that are beyond the chaperonin system, if available. Moreover, the GroEL and other chaperone (e.g. DnaK) independence in the folding of the gp23BY protein is also addressed by in vitro renaturation experiments of purified gp23 which demonstrate enhanced chaperonin-independent folding of the mutant gp23BY protein.

The chaperonin family is well known for high levels of homology across mitochondria, chloroplast, and bacterial homologues (4). Although the co-chaperonins GroES and gp31 both interact with GroEL, there is no significant sequence identity between the two co-chaperonins at the primary or predicted secondary structural levels (30, 31). However, mobile loop domains, believed to be involved in the Cpn10-GroEL interaction, have been identified in the amino-terminal region of both proteins (49, 50). Also, analogous to GroES (10 kDa), gp31 monomers are 12 kDa (10, 30) and appear to form oligomeric ring structures when over-expressed in vivo (42). However, the fact that gp31 was identified in a monomeric state in T4-infected bacteria may reflect weaker gp31 oligomerization as compared with groES (24, 37). In fact, in view of these monomer-multimer equilibria, it is not excluded that hetero-oligomeric gp31-GroES, and/or "symmetrical" gp31-GroES-GroEL complexes could form with GroEL in vivo. And it appears that T4by31 phage assembly in the presence of core proteins, approximately 50% (Table IV), is more efficient than gp23BY polyhead formation in their absence, less than 10% (Fig. 1, lane 4). This difference might explain the observation that in the absence of prohead core proteins, gp23 aggregates rather than forming polyheads in an infected bacterium (52, 61).

Although it might be supposed from experiments demonstrating a general co-chaperonin role of gp31 (19) that gp31 together with GroEL actively fold a wide spectrum of T4-coded proteins in the infected bacterium, the existence of the T4by31 mutations shows that such interactions with T4 substrates other than gp23, if they exist, are non-essential. Western blotting of cell lysates shows that both GroES and gp31 co-chaperonins are present simultaneously in phage-infected bacteria (Fig. 4) and calculations of the quantities of each Cpn10 suggest that the GroEL-gp31 interaction may be the favored Cpn60-Cpn10 interaction. Also, we have analyzed several members of the T-even bacteriophage family and have found through PCR assay that gene 31 is maintained (42). More extensive analysis (53) has revealed that gene 31 is not only maintained in 49 members of the T-even family but that the primary sequence is also highly conserved. Collectively, the data suggest gp31 has evolved to specifically address the folding needs of the T4 major capsid protein.

The T4by31-2 mutation displayed unexpected complexity, consisting of three closely linked contributing missense mutations; this reconstruction (Tables III and IV) shows why this mutation could not be mapped (43). Thirteen independently isolated T4by31 mutations have been localized as repeats of or very close to the by31-1 and by31-2 mutation sites suggesting that only a very limited number of such mutant sites exist in gene 23 (29, 43). These bypass regions display sequence homology to the T4 head vertex protein gp24 (54) (Fig. 6). It has been previously noted that, in contrast to the amber and by24 mutations, head size determining mutations (pt and ptg) and temperature sensitivity mutations (trb, ts, and cs) found in gene 23 cluster in these gp24 homology boxes (32, 54, 55). Since all of these mutant sets are thought to approach saturation, this clustering should be significant. These regions, therefore, also appear to be especially critical for the proper folding of the major capsid protein.
The locations of gene\textsuperscript{-1} and bypass\textsuperscript{31-}bypass\textsuperscript{31} place constraints on models for GroEL catalyzed folding, favoring in "chamber" folding.

The specificity of the T4 major capsid protein for the gp31 co-chaperonin may be explained in several ways according to current models for GroEL-catalyzed protein folding and chaperonin structures (7–9, 14, 56, 57). One trivial explanation is that gene 23 expression has a toxic effect on GroES forcing T4 to synthesize a "gp23 immune" GroEL-Cpn10 complex. We find this unlikely since we are able to express high levels of gene 23 constitutively from expression vectors without lethal effects on bacteria (data not shown). In another model, GroES could bar entry of gp23 to the central folding cavity of GroEL, whereas GroES engaged with the gp31 co-chaperonin might admit gp23. However, our co-immunoprecipitation experiments of infected or uninfected cell lysates with anti-GroEL antibody show that gp23, with and without gp31 present, can be co-immunoprecipitated with GroEL. This suggests that gp31 is not required for gp23 "targeting" or binding to GroEL but that it is required for the productive folding of the protein. A model in which the binding of gp31 to GroEL creates a larger folding cavity than when GroES is bound to GroEL could also explain the GroEL-gp31-specific requirement. The slightly larger gp31 might complex with GroEL while gp23 is within the folding cavity, whereas GroES would be prevented from complex formation by gp23. This model would be consistent with the relatively large (56 kDa) T4 major capsid protein and selective pressure against its size reduction. A recent crystallographic determination of the gp31 structure establishes this size increase and proposed this upper-limit size mechanism (51). However, it appears uncertain that 56 kDa is an upper limit to the GroES-GroEL complex. The ability of the GroEL-GroES complex to fold proteins larger than gp23, such as sigma 70, the bacteriophage Lambda B protein (60 kDa) or Mu H protein (64 kDa) suggests that the size of the folding cavity alone may not adequately explain the gp31 requirement (1, 20). Indeed, the fact that the lambda gpB folding requirements are less well catalyzed by the larger T4 gp31-GroEL chaperonin system (Fig. 3) argues against the folding chamber size hypothesis. If this size hypothesis is incorrect, then the gp23-gp31-GroEL chaperonin specificity results demonstrated in this paper appear to place constraints on models for GroEL catalyzed folding, favoring "chamber" folding.

An alternative explanation for these specificity results is that GroES may allow the premature release of partially folded gp23 from GroEL, whereas gp31 may stabilize the polypeptide-GroEL interaction resulting in more complete folding in the chamber. We can further speculate that GroEL bound by gp31 makes specific associations with wild-type gp23 that do not form with the GroEL-GroES complex and that these interactions enable the polypeptide to achieve native state, a view consistent with the possibly exclusive targeting of gp23 folding by gp31 in a T4 infection. In this view, the major capsid protein sequence alone may not contain all of the folding information required. Just as the bypass\textsuperscript{31} mutations impart new folding information to the gp23 polypeptide enabling chaperonin-independent folding, gp31 may engage the GroEL chaperonin in such a way that it provides some specific folding information to the wild-type gp23 molecule, e.g., by producing a more energetically folded intermediate. In fact, it is tempting to speculate that the additivity of the contributing tightly clustered bypass amino acid changes to a chaperonin-independent folding mode corresponds to efficient localized GroEL chaperonin action on these portions of the polypeptide substrate. In this view, the role of the GroEL-gp31 chaperonin system may not only be to shift the folding equilibrium away from aggregation but through specific GroEL-gp31-gp23 interactions to foster an environment that allows for proper folding of specific refractory sequences or local structures. Although the three-dimensional structure of the T4 major capsid protein is not determined, it has been shown to undergo a remarkably large structural change which extends to the secondary level, following its assembly preparatory to DNA packaging (Fig. 6); thus, it is assembled in a metastable state in the polyhead (32, 58). Whether this is related to the specific co-chaperonin assembly requirement remains to be determined.

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REFERENCES

1. Tilly, K., and Georgopoulos, C. (1982) J. Bacteriol. 149, 1082–1088
2. Tilly, K., Murielde, H., and Georgopoulos, C. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 1629–1633
3. Henrici, P. J., and Hartl, F. U. (1993) Annu. Rev. Biophys. 22, 349–384
4. Zeljztra-Rylls, J., Fayet, O., and Georgopoulos, C. (1991) Annu. Rev. Microbiol. 45, 301–325
5. Fayet, O., Ziegelhoffer, T., and Georgopoulos, C. (1989) J. Bacteriol. 171, 1379–1385
6. Viitanen, P., Gatenby A., and Lorimer, G. H. (1992) Protein Sci. 1, 363–369
7. Braig, K., Simon, M., Furuwa, F., Hannfeld, J. F., and Horwich, A. L. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3978–3982
8. Xu, Z., Horwich, A. L., and Sigler, P. B. (1997) Nature 388, 741–750
9. Braig, K., Otwinowski, Z., Hegde, R., Bovisert, D. C., Joachimiak, A., Horwich, A. L., and Sigler, P. B. (1994) Nature 371, 578–586
10. Chandraexkhar, G. N., Tilly, K., Woolford, C., Hendrix, R., and Georgopoulos, C. (1986) J. Biol. Chem. 261, 12414–12419
11. Saibil, H., Dong, Z., Wood, S., and auf der Mauer, A. (1991) Nature 353, 25–36
12. Chen, S., Roseman, A. M., Hunter, A. S., Wood, S. P., Burston, S. G., Ranson, N. A., Clarke, A. R., and Saibil, H. R. (1994) Nature 371, 261–264
13. Hunt, J. F., Weaver, A. J., Landry S. J., Gierasch, L., and Deisenhofer, J. (1996) Nature 379, 37–45
14. Fenton, W. A., Kashi, Y., Furtak, K., and Horwich, A. L. (1994) Nature 371, 614–619
15. Saibil, H. (1996) Structure 4, 1–4
16. Mizobata, T., Akiyama, Y., Ito, K., Yamamoto, N., and Kawata, Y. (1992) J. Biol. Chem. 267, 17775–17779
17. Gray, T. E., and Fersht, A. R. (1993) J. Mol. Biol. 232, 1197–1207
18. Clark, A. C., and Frieden, C. (1997) J. Mol. Biol. 268, 512–525
19. van der Vies, S. M., Gatenby A., and Lorimer, G. H. (1992) Proc. Natl. Acad. Sci. U. S. A. 90, 3978–3982
20. Karam, J. D. (ed) (1994) Molecular Biology of Bacteriophage T4, (Karam, J. D., ed) pp. 218–358, American Society for Microbiology, Washington, D.C.
21. Crohnse, T., Swoffield, J. C., and Brown, A. J. P. (1992) J. Mol. Biol. 228, 7–12
22. Thanaraj, E. A., and Argos, P. (1996) Protein Sci. 5, 1564–1612
23. Masson, J. M., and Miller J. H. (1986) Gene (Amst.) 47, 179–183
24. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1996) Methods Enzymol. 185, 60–89
25. Zondio, J., Fisher, K. E., Lin, A., Ducote, K. R., and Eisenstein, E. (1995) Biochemistry 34, 10334–10339
26. Ho, S. N., Hunt, H. D., Horton, R., Pullen, J., and Pease, L. E. (1989) Gene (Amst.) 77, 51–59
27. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
28. Takano, T., and Kakefuda, T. (1972) Nature 239, 232–238
29. Black, L. W., Showe, M., and Steven, A. C. (1994) in Molecular Biology of Bacteriophage T4, (Karam, J. D., ed) pp. 218–358, American Society for Microbiology, Washington, D.C.
Substrate Mutations That Bypass a Specific Cpn10 Chaperonin Requirement for Protein Folding
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