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Cooperation of GroEL/GroES and DnaK/DnaJ heat shock proteins in preventing protein misfolding in *Escherichia coli*

(protein aggregation/protein folding/chaperones/*rpoH* mutants)

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ABSTRACT **Newly synthesized proteins aggregate extensively in *Escherichia coli rpoH* mutants, which are deficient in the heat shock proteins (hsp). Overproduction of either GroEL and GroES or DnaK and DnaJ prevents aggregation. If expressed together, the four hsp are effective at physiological concentrations. Our data suggest that the GroEL and GroES proteins and the DnaK and DnaJ proteins have complementary functions in the folding and assembly of most proteins.**

The folding and assembly of cellular polypeptides is thought to require molecular chaperones as accessory factors (for recent reviews, see refs. 1–4). The requirement has been demonstrated for several specific proteins (5–8) and is suggested by the association of chaperones with newly formed polypeptides (9, 10) or with polypeptides that are translocating across organelle membranes (11, 12). The chaperones either belong to the heat shock proteins (hsp) or are closely related to them (1). To determine the specificity of chaperones and their target proteins, we have studied the properties of proteins synthesized in hsp-deficient *Escherichia coli*.

E. coli rpoH mutants lack the heat shock σ subunit and are unable to induce hsp. Proteins aggregate extensively in *rpoH* mutants at elevated temperature (13, 14), suggesting that one or more hsp allows proteins to achieve their normal soluble conformation. Protein aggregation in *rpoH* mutants was dependent on protein synthesis, implying that the correct folding and/or assembly of newly synthesized polypeptides was impaired (13). Similarly, proteins of *rpoH* mutant cells labeled at permissive temperature remained largely soluble after transfer to elevated temperature (unpublished observation). Two families of hsp, hsp60 and hsp70 (60- and 70-kDa hsp) have been implicated in protein folding and assembly. The *E. coli* hsp60, GroEL, binds newly formed polypeptides (9) and acts in concert with GroES to promote proper assembly of a variety of proteins, including phage structural proteins (5–7). The hsp70 analogue of *E. coli*, DnaK, along with DnaJ and GrpE, is required for replication of phage λ and of plasmids P1 and F (reviewed in ref. 15). DnaK and DnaJ stimulate replication of λ by dissociating the λP protein from a λori complex (15) and of P1 by converting P1 RepA dimers to the active monomeric form (8). DnaK also activates *in vitro* the *E. coli* replication initiator protein DnaA (16). In eukaryotes, hsp70 and cognate proteins are associated with nascent polypeptide chains (10) and, along with hsp60, are required for proper folding of proteins imported into mitochondria (11, 12).

In this manuscript, we report the effects of expressing four hsp—GroEL, GroES, DnaK, and DnaJ—in *rpoH* mutants. We find that, at high concentrations, either GroEL/GroES or DnaK/DnaJ prevents aggregation of newly synthesized proteins, while at physiological concentrations the expression of all four hsp is required.

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MATERIALS AND METHODS

Strains and Plasmids. The *E. coli* strains used are listed in Table 1. Ω585 and Ω587 are *thr*$^+$ derivatives of Ω419 to which the *groEL100* or *groES30* alleles were introduced by P1 transduction via their linkage with the Tn*10* marker. Plasmid pKJ, described as pJM2 by McCarty and Walker (22), contains the *dnaK* operon placed under control of the *lac* promoter. Plasmids pK (having only the *dnaK* gene) and pJ (having only the *dnaJ* gene) are pKJ derivatives obtained by deletion of an appropriate restriction fragment. pSL expresses the *groE* operon from P*lac*. It was constructed by inserting the *groE* operon-containing *Eco*RI restriction fragment, trimmed upstream with BAL-31 nuclease, into *Sma* I/*Eco*RI-digested pUC19. pS and pL are derivatives of this plasmid having the *groEL* and *groES* genes, respectively, inactivated by deletion of appropriate restriction fragments. The *lacI*^{q}-containing plasmid was constructed by insertion of an *Eco*RI restriction fragment with the *lacI*^{q} gene into pACYC184.

Media and Growth Conditions. Strains were grown in LB medium at 30°C to mid-logarithmic phase. The cells were then incubated at 42°C or at 30°C for 1 hr. For plasmid-containing cells, ampicillin (100 μg/ml) was added to the medium. Cells having *rpoH* null allele were grown at 18°C, rather than 30°C, before transfer to 42°C.

Preparation and Fractionation of Cell Lysates. Cells were chilled on ice, collected by centrifugation, and disrupted by sonication (four or five 10-sec bursts) on ice in Laemmli loading buffer (23) without SDS and containing phenylmethylsulfonyl fluoride (200 μg/ml). The extent of cell disruption was monitored by phase-contrast microscopy. Usually, <0.1% of cells remained intact after sonication. The cell lysates were centrifuged in a Microfuge 11 (Beckman) at 13,500 rpm for 2 min. SDS (final concentration, 1%) was added to the supernatant. The pellet was rinsed once with excess buffer, centrifuged, and resuspended in half the original volume of SDS-containing loading buffer. Before electrophoresis, the samples were heated in a boiling water bath for 1–2 min. Equal volumes of soluble and insoluble protein fractions were separated on SDS/10% PAGE (23) and stained with Coomassie blue.

RESULTS

Overproduction of Either GroEL and GroES or DnaK and DnaJ Prevents Protein Aggregation. To permit expression of hsp genes in *rpoH* mutants, we used plasmids carrying the

Abbreviation: hsp, heat shock protein(s).

Table 1. *E. coli* strains

Strain	Relevant genotype	Source or ref.
SC122	F⁻ *lac(am) trp(am) pho(am) supC^{ts} strA mal(am)*	17
K165	SC122 *rpoH165*	17
CAG9301	F⁻ *rpoH120*::*kan zhg21*::Tn*10*	18
MC4100	F⁻ *araD139* Δ(*argF-lac*)*U169 rpsL relA flbB deoC ptsF rbsR*	19
KY1603*	MC4100 *rpoH30*::*kan zhf50*::Tn*10 suhX401* (λ*pfl3*-P*rpoD$_{hs}$-lacZ*)	20
KY1617†	MC4100 *rpoH30*::*kan zhf50*::Tn*10 suhX302 suhY421* (λ*pfl3*-P*groE-lacZ*)	20
Ω392	F⁻ *sup^{o} galKTE*-P$_R$-λcI857*indl*-P$_L$-*Nam7,53*	21
Ω394	Ω392 *thr*::Tn*10*	21
Ω419	Ω392 *thr*::Tn*10 dnaK756*	21
Ω425	Ω392 *groEL100 groES*⁺::Tn*10*	21
Ω427	Ω392 *groEL*⁺ *groES30*::Tn*10*	21
Ω585	Ω392 *dnaK756 groEL100 groES*⁺::Tn*10*	This work
Ω587	Ω392 *dnaK756 groEL*⁺ *groES30*::Tn*10*	This work

*Referred to in the text as R40.
†Referred to in the text as R42.

groEL–groES or *dnaK–dnaJ* operons under *lac* promoter control. Introduction of the *groEL–groES* plasmid (pSL) into *rpoH165* hosts largely eliminated protein aggregation (Fig. 1*A*). Both GroEL and GroES were required; a plasmid expressing only the former (pL) failed to prevent aggregation (Fig. 1*A*). Overexpression of GroES alone was likewise ineffective (data not shown).

Overexpression of DnaKJ (plasmid pKJ) also prevented protein aggregation in the *rpoH165* mutant (Fig. 1*B*). Both DnaK and DnaJ were required; a plasmid expressing only DnaK (pK) or DnaJ (pJ) failed to maintain protein solubility (Fig. 1*B*). We cannot exclude the possibility that GrpE, present at basal levels in *rpoH* strains, participates in this reaction.

Plasmids pSL and pKJ express large quantities of GroELS and DnaKJ, respectively, from the P*lac* promoter (see Fig. 1). To determine whether such overproduction was necessary to prevent aggregation, we modulated the activity of P*lac* by introducing a compatible, multicopy plasmid bearing the *lacI^{q}* gene. Glucose was added to the medium to further repress expression. The combined effects of *lac* repressor and glucose-induced catabolite repression were insufficient to inhibit completely the expression of GroELS or DnaKJ, and the residual levels of these proteins were equal to or higher than those in wild-type cells (compare Figs. 1 and 2). However, these concentrations were insufficient to prevent aggregation in the *rpoH165* mutant (Fig. 2). Activation of P*lac* with isopropyl β-D-thiogalactopyranoside and omission of glucose from the medium induced overexpression of GroELS and DnaKJ and blocked aggregation (Fig. 2). We conclude that the efficiency of GroELS or DnaKJ in preventing aggregation is low and that significant solubilization of newly synthesized proteins requires overproduction of either hsp pair.

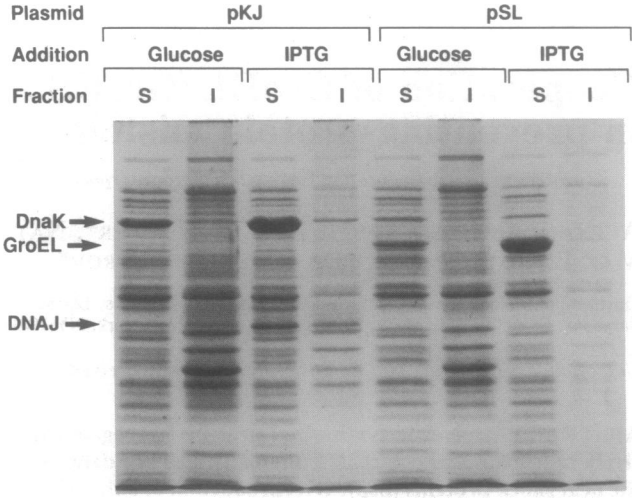

FIG. 2. Overexpression of *groESL* or *dnaKJ* is required to prevent aggregation. *rpoH165* cells with plasmids bearing *groE–groES*, or *dnaK–dnaJ*, as well as a compatible plasmid coding for *lacI^{q}* repressor, were grown in LB medium supplemented with either isopropyl β-D-thiogalactopyranoside or glucose at 30°C and transferred to 42°C for 1 hr. Soluble (lanes S) and insoluble (lanes I) fractions were obtained as described and separated by SDS/PAGE.

GroELS and DnaKJ Act Synergistically to Prevent Protein Aggregation. We suspected that GroELS and DnaKJ might act synergistically, and we tested this possibility by using a different *rpoH* mutant and its derivatives. The *rpoH* deletion strain (*rpoH30*), isolated by Kusukawa and Yura, is inviable at temperatures exceeding 20°C (18). Selection at 30°C

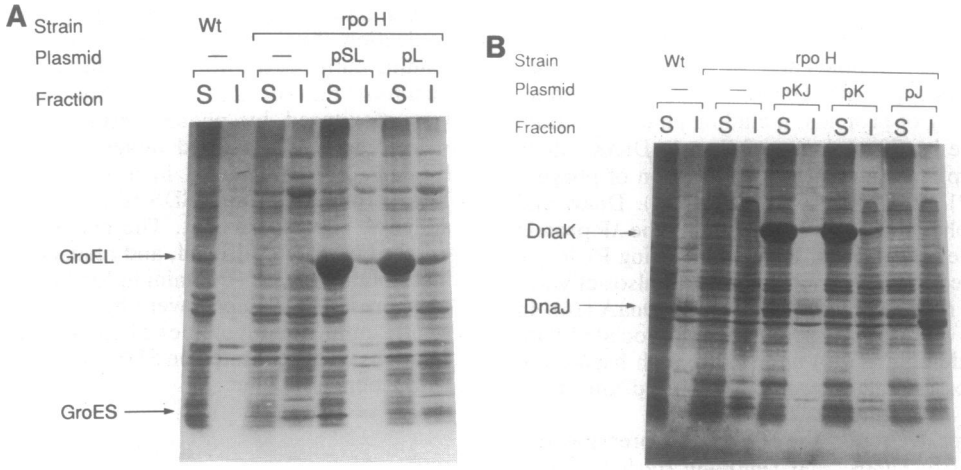

FIG. 1. Expression of *groESL* (*A*) or *dnaKJ* (*B*) prevents protein aggregation in *rpoH165* mutant. Wild-type (wt) (SC122) or *rpoH* (K165) cells were grown in LB medium at 30°C and transferred to 42°C for 1 hr. Where indicated, *rpoH* strains contained plasmids bearing *groEL–groES* (pSL), *groEL* alone (pL), *dnaK–dnaJ* (pKJ), *dnaK* alone (pK), or *dnaJ* alone (pJ) under the control of the *lac* promoter. Soluble (lanes S) and insoluble (lanes I) fractions were obtained as described and separated by SDS/PAGE. Note that insoluble fractions were loaded on the gel in twice the amount of the corresponding soluble fraction.

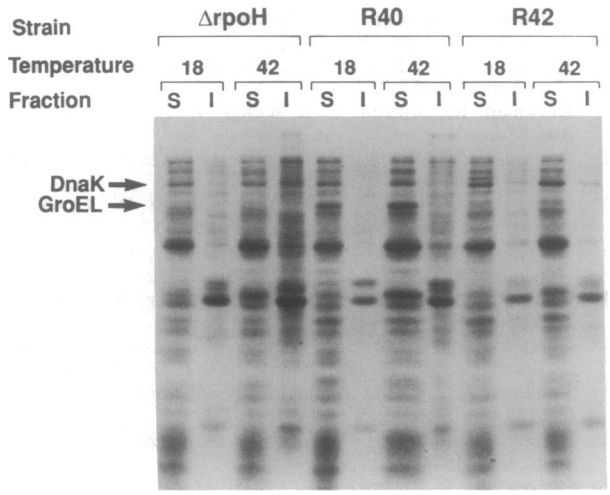

FIG. 3. Protein aggregation in the *rpoH* deletion strain and in the revertant strains R40 and R42. Cells of the *rpoH120* mutant and revertants R40 and R42 were grown in LB medium at 18°C and transferred to 42°C for 1 hr. Soluble (lanes S) and insoluble (lanes I) fractions were obtained as described and separated by SDS/PAGE. Strains R40 and R42 are isogenic. Their parental strain carrying the *rpoH30* mutation without suppressor mutations was not available to us. Therefore, we used the nonisogenic *rpoH120* deletion strain (CAG9301; see Table 1) as a control for this experiment.

yielded the partial revertant R40, which expresses *groEL–groES* constitutively from a σ^{32} subunit-independent promoter (20). From a revertant isolated at 30°C, selection for colony formers at 42°C yielded R42, which, additionally, expresses *dnaK–dnaJ* constitutively (20). R40 and R42 express the hsp at levels approaching those of the wild-type strains at 42°C. The aggregation of proteins in the *rpoH* null mutant at 42°C was even more extensive than in *rpoH165* (Fig. 3) and was blocked by plasmid pLS or pKJ (data not shown). Constitutive expression of GroELS partially alleviated protein insolubility, although significant aggregation persisted in R40 (Fig. 3). The expression of GroELS and DnaKJ in the R42 revertants almost completely blocked aggregation (Fig. 3). These results indicate that GroELS and DnaKJ act synergistically at physiological concentrations to ensure proper folding and/or assembly of proteins. Furthermore, since R42 cannot express high levels of the other σ^{32} subunit-dependent hsp, a role for these proteins in this reaction may be marginal.

Protein Aggregation in *groE/dnaK* Double Mutants. Unlike the *rpoH* mutation, mutations in *groEL, groES, dnaK* (Fig. 4), or *dnaJ* (data not shown) failed to induce protein aggregation. In contrast, strains mutant in both *dnaK* and *groEL* or *dnaK* and *groES* contained significant levels of insoluble polypeptides. We presume that the lack of phenotype for the single mutants reflects both the leakiness of the mutation and the ability of GroELS and DnaKJ to substitute partially for each other. This experiment confirms the role of GroELS and DnaK in ensuring proper protein conformation and, additionally, shows that they cannot be efficiently replaced by other hsp.

DISCUSSION

Our previous work demonstrated that newly synthesized proteins aggregate and form inclusion bodies in *rpoH* mutant cells, which are deficient in the hsp (13). We suggested that this aggregation resulted from interactions between misfolded proteins and that the hsp prevent these interactions. The evidence presented in this manuscript indicates that GroELS and DnaKJ are responsible for maintaining the solubility of *E. coli* proteins (Figs. 1 and 2). The failure of the majority of the bacterial proteins to acquire native conformation may explain the conditional lethality of *groESL* (24), *dnaK* (25), and *dnaJ* (26) mutants.

We find no indication of hsp specificity in maintaining protein solubility. For example, GroELS expressed at physiological concentrations partially prevents protein aggregation without apparent selectivity for different proteins, as judged by band patterns on SDS/polyacrylamide gels (Fig. 3). Our data suggest that DnaKJ and GroELS act in the same folding/assembly pathway rather than in parallel pathways. This is in contrast to assays for folding or disaggregation of particular proteins by hsp *in vitro*, which indicate a high degree of specificity. For example, the correct initiation of λ DNA replication requires DnaK, DnaJ, and GrpE (27),

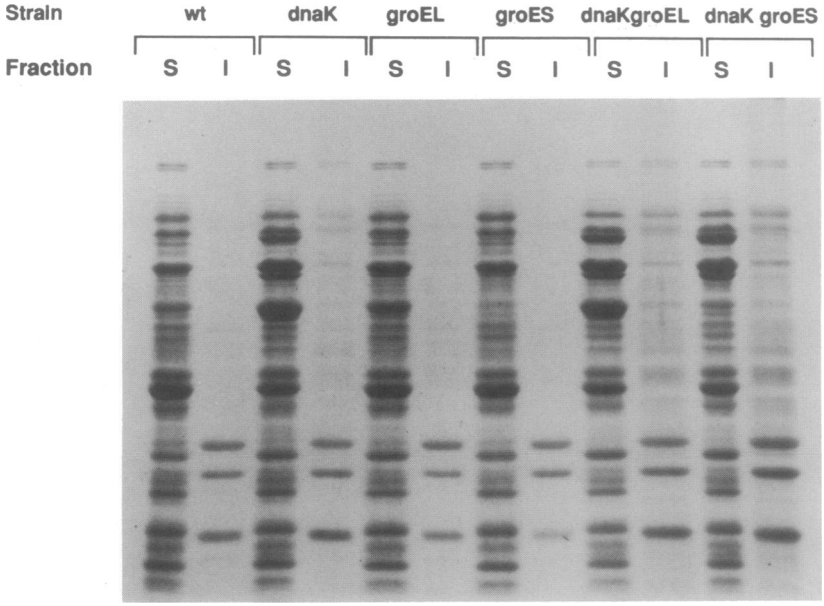

FIG. 4. Protein aggregation in *dnaK, groEL, groES*, and double-mutant cells. Wild-type (wt) or mutant cells were grown in LB medium at 30°C and transferred to 42°C for 1 hr. Soluble (lanes S) and insoluble (lanes I) fractions were obtained as described and separated by SDS/PAGE. Strains used for this experiment are listed in Table 1.

whereas GroELS specifically reactivates denatured ribulose-bisphosphate carboxylase (5).

Our experiments also indicate that GroELS and DnaKJ act synergistically to prevent aggregate formation in *rpoH* mutants (Fig. 3). Either hsp pair alone is inefficient and must be overexpressed to block aggregation (Fig. 2), which is indicative of stoichiometric rather than catalytic action of the hsp. These data are in good agreement with the recent demonstration of an interplay of DnaK and GroEL (with their respective accessory proteins) in protein refolding *in vitro* (28). It is likely that the two classes of bacterial hsp act successively in the folding/assembly pathway of most cellular proteins. Our data indicate such cooperation in *E. coli*, interactions between hsp60 and hsp70 in the import of proteins into yeast mitochondria have been also noted (12). The recent report of a hsp60-like protein in a eukaryotic cytosol suggests that functional interactions between hsp60 and hsp70 may be a general requirement for proper folding and assembly of proteins (29).

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