Purification and Properties of the groES Morphogenetic Protein of Escherichia coli*

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The morphogenesis of λ proheads is governed by the products of at least four bacteriophage-coded genes (B, C, E and Nu3) and two host-coded genes (groES (mopB) and groEL (mopA)). Earlier genetic experiments indicated that the phenotypes of some of the groES* mutations could be suppressed by mutations in the groEL gene, suggesting an interaction between the two groE proteins in vivo (Tilly, K., and Georgopoulos, C. P. (1982) J. Bacteriol. 149, 1082–1088). The M, 15,000 groES protein was overproduced and purified to homogeneity by a combination of gel filtration on a Sephadryl S-300 column and acrylamide gel electrophoresis. Both gel filtration on an AcA34 size-exclusion column and glycerol gradient centrifugation indicate that the groES protein possesses an oligomeric structure of M, 80,000. In agreement, electron microscopic pictures of the purified groES protein show that it possesses a symmetrical ring-like structure. The sequence of the first five amino acids and the overall composition of the purified protein match those predicted by the nucleotide sequence of the groES gene. The following results implicate a physical association between the groES and groEL proteins in vitro. (a) The groES protein inhibits the weak ATPase activity of the groEL protein, with a maximal effect seen at 1:1 molar ratio; (b) the two proteins co-sediment during glycerol gradient centrifugation in the presence of ATP and Mg2+; and (c) the groES protein binds specifically to a groEL-affinity column. These results help explain why mutations in either of the groE genes exhibit similar phenotypes with respect to both λ and bacterial growth.

The Escherichia coli groE genes were originally discovered because mutations in them blocked productive growth of bacteriophages λ and T4 (1, 2). The two genes were subsequently shown to form an operon with the structure promoter groES-groEL, which is subject to heat shock regulation (3–5) and maps at 93.5 min on the E. coli genetic map (6). The groES and groEL genes have products that have been shown to be necessary for proper λ prohead assembly and are proteins of M, 15,000 and M, 65,000, respectively (7–9). The groEL protein has been purified to homogeneity (10–12). In its native form the groEL protein is a decameric protein, with its subunits arranged in a double ring with 7-fold symmetry (10, 11). This complex sediments at S 25 and exhibits a weak ATPase activity (11, 12). The groEL protein seems to act in λ prohead assembly at a step involving the bacteriophage-coded minor head protein B (13). It has recently been shown that a functional groEL protein is necessary for the oligomerization of the B protein into a dodecameric structure (14). The B protein dodecamer is located at the vertex of the λ head to which the λ tail attaches to form a mature virion (15). A direct role for the groES protein in the oligomerization of the B protein has been implicated but has not been conclusively demonstrated yet.

Both the groES and groEL proteins are also required for growth of bacteriophage T5 (16, 17). Surprisingly, the two proteins are necessary for tail but not head assembly (17). Evidence exists that the groEL protein is also required for proper bacteriophage T4 head assembly at the level of action of the bacteriophage-coded 51 protein (2, 16). However, none of the groES* mutants tested so far blocks T4 morphogenesis, suggesting that the groES protein may not be essential for T4 growth (16). Mutations in both the groES and groEL genes also render the bacteria temperature sensitive for colony formation at 43°C, suggesting that they are essential for growth, at least at high temperature (18, 19). Although the mechanism of action of the groES and groEL proteins is not known, genetic evidence has been presented demonstrating that the two proteins interact functionally in vitro (16).

In this communication, we describe the purification and characterization of the groES protein. Furthermore, we show that the two purified groES proteins interact functionally in vitro.

MATERIALS AND METHODS

Buffers—Lysis buffer: 50 mM Tris-HCl (pH 8.0), 10% (w/v) sucrose, and 5 mM 2-mercaptoethanol. Buffer A: 50 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 5 mM 2-mercaptoethanol, 1 mM EDTA, 0.05% (v/v) Triton X-100, and 0.1 M ammonium sulfate. Buffer B: 50 mM Tris-HCl (pH 8.0), 10% (v/v) glycerol, 5 mM 2-mercaptoethanol, 5 mM EDTA, 0.5% (v/v) Triton X-100, and 1 M KCl. Buffer C: 50 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 5 mM 2-mercaptoethanol, and 1 mM EDTA. Buffer D: buffer C with 50 mM KCl. Buffer E: 50 mM Tris-HCl (pH 8.0), 5% (v/v) glycerol, 2 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1 M KCl. Buffer F: 100 mM Tris-HCl (pH 8.0), 50 mM 2-mercaptoethanol, 50 mM EDTA, 2 mg/ml egg white lysozyme, 1 mM ammonium sulfate, and 0.18 M spermidine hydrochloride.

Construction of Plasmids for Overproduction of the groEL and groES Proteins—The groES gene was previously shown to be located on an 8.0-kb EcoRI fragment of E. coli DNA (7). This fragment was purified from the λ groE transducing phage W5 (7) and ligated to EcoRI nucleases-digested pBR322 DNA. The resulting plasmid, containing the complete groEL and groES genes, was called pLS1. DNA from

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pLS1 was partially digested with the Sau3A restriction nuclease and ligated into the unique BanHI site of plasmid pH8 DNA (20). From this ligation a plasmid containing an approximately 1-kb bacterial DNA insertion including the complete groES gene, its promoter, and a small portion of the groEL gene was isolated and called pS4.

**Purification of the groEL Protein.—**The groEL protein was purified according to the procedure of Hendrix and Ishihama et al. according to the procedure described in Ref. 22. The supernatant was ligated into the unique BamHI site of plasmid pJB8 DNA (20). From was precipitated with ammonium sulfate (35-50% saturation), centrifuged at 30,000 rpm for 45 min at 2 °C in a Beckman R35 rotor, resuspended in buffer B, and passed through a Bio-Gel A-1.5m column (Bio-Rad) equilibrated in the same buffer.

**Glycerol Gradient Sedimentations—**Purified groEL and groES proteins were incubated at 30 °C for 30 min in 50 mM Tris-HCl (pH 8.0), 5% (v/v) glycerol, 50 mM KCl, 10 mM MgCl2, 5 mM 2-mercaptoethanol, and 0.2 mM ATP (25-μl total reaction volume). At the end of incubation, the entire mixture was loaded onto a 0.75-m1 10-30% (v/v) glycerol gradient in the same buffer and centrifuged at 38,000 rpm for 2 h at 4 °C in a Beckman SW 60 Ti rotor. Approximately 15-20 fractions (1 drop) were collected after puncturing the tube at the bottom, 15 μl of 4× SDS-sample buffer were added, the samples were electrophoresed through 12.5% SDS-polyacrylamide gels, and the gels were stained with silver nitrate (23).

**Coupling of the groEL Protein to Affi-Gel 10 Matrix—**Coupling of the groEL protein to Affi-Gel 10 matrix (Bio-Rad) was carried out according to Formosa et al. (24). Approximately 600 μg of groEL protein (in a 500-μl volume) were dialyzed into the coupling buffer (100 ml), sodium bicarbonate (pH 8.0) with 0.5 M NaCl and agitated with 0.6 ml of Affi-Gel 10 matrix for 24 h at 4 °C. At the end of incubation, the mixture was centrifuged at 4000 rpm for 5 min at 4 °C in a Beckman JA-20 rotor to collect the matrix, and the remaining active material of the cell extract and to fractionate through a Bio-Gel A-1.5m gel filtration column (see "Materials and Methods"). The groES protein-containing peak fractions were pooled, dialyzed overnight against two changes of 5000 ml of buffer C (Step III, lane 4), and passed through a hydroxylapatite column (1 × 7 cm, Bio-Rad) equilibrated with buffer C. The column was washed with 2 column volumes of buffer C with 25 mM phosphate (pH 7.4) and 2 column volumes of buffer C with 50 mM phosphate (pH 7.4). Fractions containing the groES protein, eluting in the second wash, were pooled (Step IV, lane 5) and passed over a DE52-cellulose column (3 × 15 cm, Whatman) equilibrated in buffer D, washed with 2 column volumes of buffer D with 100 mM KCl, buffer D with 150 mM KCl, and finally eluted with a linear gradient of 150-350 mM KCl in buffer D (100 ml). Approximately 0.5 M fractions were collected. The fractions eluting in the 200-250 mM KCl range contained groES protein over 80% pure (Step V, lane 6). These fractions were pooled, concentrated, and dialyzed against buffer E. The concentrated sample (about 0.5 ml) was layered onto a 38-ml 10-30% (v/v) linear glycerol gradient in buffer E and centrifuged in a Beckman SW 27 rotor at 24,000 rpm for 8 h at 4 °C. Fractions were collected from the bottom, and fractions exhibiting at least 90% purity of the groES protein were stored at −70 °C (Step VI, lane 7). The arrow points to the position of the groES protein.

**Electron Microscopy—**GroES protein was placed on grids with carbon-coated Parlodion films, stained with a drop of uranyl formate (0.5%, freshly made), and examined in a Philips EM300 microscope at an accelerating voltage of 80 kV. The very small size of the groES protein molecules meant that negative stain did not accumulate around the protein unless it was used at such a high concentration that it formed uninterpretable mounds on the grid. This problem was solved by including virions of bacteriophage P22 with the groES protein. The negative stain accumulates around the virions and forms a gradient of decreasing thickness at increasing distance from the virion. Under these conditions groES protein can be used at more moderate concentrations which allow visualization of individual molecules.

**RESULTS**

**Overproduction of the groES Protein—**In order to maximize the levels of groES protein in cell lysates, we both increased the gene copy and took advantage of the response of the groE promoter to heat shock. We found that growing B178 (pLS1) cells in Luria broth (7) at 30 °C until they reached an optical density of 0.6 at 600 nm and then shifting to 42 °C for an

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1 The abbreviation used is: SDS, sodium dodecyl sulfate.
protein (2 mg/ml) was loaded on an AcA gel AcA34 sizing column. In a parallel experiment, standard gel filtration marker proteins (Sigma) (1) yeast alcohol dehydrogenase (M, 150,000), (2) bovine serum albumin (M, 66,000), (3) bovine erythrocyte carbonic anhydrase (M, 29,000), and (4) horse heart cytochrome c (M, 12,400) were mixed (2.5 mg of protein each), and the relative positions of their elution were determined by monitoring absorbance at 280 nm. V₀ and Vₑ were determined by the positions of dextran blue (Sigma) and Vitamin B₁₂ (Sigma), respectively.

Additional 3-h growth period before harvesting yielded 10–20 times more groES protein than growing cells without the plasmid at 30 °C.

No functional biological assay is available for the groES protein yet, so its purification was monitored by following the SDS-polyacrylamide gel profiles of the various fractions. This was possible because the positions of the groES protein in both one- and two-dimensional polyacrylamide gels are known (9). That the overproduced groES protein is functional in vivo was shown by the fact that mutant groES30 bacteria transformed with the pLS1 plasmid both supported bacteriophage λ growth and became temperature resistant at 43 °C (data not shown).

Purification of the groES Protein—The purification of groES protein, described in detail in the legend to Fig. 1, involved seven successive purification steps. Fig. 1 shows an SDS-polyacrylamide gel of material from the different stages of the purification. The final yield of groES protein, as judged by Coomassie Blue staining in two-dimensional gels, was between 10 and 20%.

Purity, Molecular Weight, Amino Acid Composition, and Amino-terminal Sequence—The purified groES protein migrated as a single band when analyzed by electrophoresis on 12.5% SDS-polyacrylamide gels (Fig. 1). Judging from a densitometric analysis of the Coomassie Blue staining pattern, our purified groES protein preparations were at least 85% pure (data not shown). Occasionally, a M, 88,000 polypeptide was observed as a minor contaminant, but it never amounted to more than 5% of the total protein.

On 12.5% SDS-polyacrylamide gels, the purified groES protein migrated at a position close to that expected for a M, 15,000 protein, in agreement with previously published observations (9). However, when purified groES protein was passed through an Ultrgel AcA34 (LKB) sizing column, the bulk of the protein eluted as if it were larger than bovine serum albumin (M, 66,000), corresponding to the position expected for a M, 80,000 protein (Fig. 2). The Stokes radius of the multimeric form of the groES protein was calculated to be 39.5 ± 0.5 Å according to the method of Siegel and Monty (25). These results indicate that the native form of groES is a multimer. Similarly, during glycerol gradient centrifugation, groES protein sedimented at 4.5 S, a position expected for a M, 70,000 protein, confirming the above results (data not shown).

Fig. 3 shows an electron micrograph of purified groES protein negatively stained with uranyl formate. The molecules appear to be a homogeneous population of donut-shaped structures. The resolution limit of this method does not allow individual subunits to be visualized, but the shape of the oligomer suggests strongly that the subunits are arranged with rotational symmetry around an axis through the center of the donut. The diameter of the molecules is ~8 nm, and there appears to be a small hole (~2 nm) in the center. The molecules can be seen only in areas of very thin negative stain; comparison of this property to the properties of similar molecules of known thickness suggests that the groES protein molecules are quite thin (<4 nm). The properties of groES protein revealed by electron microscopy are all consistent with the properties deduced from the biophysical measurements described above.

Table I shows that the amino acid composition of the purified groES protein agrees well with that of the amino acid composition of the groES protein predicted from its DNA
The amino acid analysis was done in the laboratory of Dr. W. Gray, Department of Biology, University of Utah. The purified protein was hydrolyzed with redistilled 6 N HCl in vacuo for 20 h at 105 °C, and its composition was determined using a Beckman 121 amino acid analyzer (29). The nucleotide sequence of the groES gene is from our unpublished work.

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<tr>
<th>Amino acid</th>
<th>Chemical analysis</th>
<th>DNA sequence</th>
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<td>Arginine</td>
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Amino-terminal sequence: Met-Asn-Ile-Arg-Pro

* Not determined.

FIG. 4. Inhibition of the groEL's ATPase activity by the purified groES protein. About 1 μg of purified groEL protein was incubated on ice with varying amounts (0.05–5 μg) of purified groES protein (Step VI) or bovine serum albumin in 15 min in the assay buffer (see "Materials and Methods"). At the end of preincubation, reactions were started by the addition of [32P]ATP and incubation at 37 °C. The amount of ATP hydrolyzed was estimated as described under "Materials and Methods." 100% activity represents the hydrolysis of 50% of input ATP under the assay conditions used (about 25,000 dpm of ADP produced). ATPase activity of groEL protein is either in the presence of groES protein (- - -) or bovine serum albumin (○ ○ ○). Molar ratio refers to subunits of groES/groEL.

DISCUSSION

The purification of the E. coli morphogeneic protein groES was aided by cloning the groES gene on a multicopy plasmid. The resulting increased number of gene copies/cell plus the strength and heat shock induction of the groE promoter resulted in a large overproduction of the groES protein at 42 °C. Starting with such extracts and following standard protein purification procedures, the protein was purified to at least 85% homogeneity. Since no enzymatic activity of the groES protein is known, we monitored the purification by following the presence of the M, 15,000 polypeptide after SDS-polyacrylamide gel electrophoresis. The identity of the groES protein with our purified protein was established by demonstrating that (a) the sequence of the first five amino-terminal amino acids and (b) the overall amino acid compo-
The nucleotide sequence of the cloned groES gene.

The nucleotide sequence of the cloned groES gene suggests peptide on SDS-polyacrylamide gels (7-9). It chromatographs at the position of the purified protein agreed with those predicted by the DNA sequence of the cloned gene. The protein is shown to possess an oligomeric structure, most likely consisting of 6 subunits. The ring-like structure of the groES protein under the conditions used for in vitro, although the resolution is not good enough to allow us to determine the symmetry and the actual number of subunits. The multimeric structure of the groES protein appears to be extremely stable, inasmuch as it is not disrupted in the presence of either 4 M urea or 600 mM KCl (data not shown).

Previously we presented in vivo data demonstrating a functional interaction between the groES and groEL proteins (16). This was accomplished through the isolation of extragenic suppressors of the groES619 mutation and demonstration that some of them are located in the groEL structural gene (16). In this paper, we have demonstrated a physical association between groES and groEL proteins, E, into a prohead structure. The dodecameric B protein to initiate correct polymerization of the major capsid protein, E, into a prohead structure. The presence of active groEL protein has been shown to be absolutely essential for the oligomerization of the B protein (14, 26). This structure is thought to interact with the χN nucleation protein to initiate correct polymerization of the major capsid protein, E, into a prohead structure. The dodecameric B protein structure is found located at the proximal vertex, where the assembled tail attaches to the head. The presence of active groEL protein was never seen to exceed 60%, and it was achieved at a 1:1 molar ratio. Similarly, only a fraction of the groES protein cosedimented with groEL protein on glycerol gradients under the conditions used for incubation. The following reasons could account for the apparent weakness of the interaction between the two proteins in vitro: (i) the groEL, groES, or both proteins may lose part of their activity during the purification process, and only active proteins can interact with one another, (ii) we may not have found the optimal incubation conditions necessary for an increased association of these proteins, or (iii) the association between groES and groEL may be inherently weak.

Recent experiments indicate that one of the key early steps in the correct assembly of λ proheads is the oligomerization of a minor subunit of the head, the B protein, into a dodecameric structure, possessing an asymmetric dumbbell shape (14, 26). This structure is thought to interact with the χN nucleation protein to initiate correct polymerization of the major capsid protein, E, into a prohead structure. The dodecameric B protein structure is found located at the proximal vertex, where the assembled tail attaches to the head. The presence of active groEL protein has been shown to be absolutely essential for the oligomerization of the B protein (14). Since (a) the phenotypes of groES and groEL mutations on bacteriophage λ prohead assembly are identical and (b) the groES and groEL proteins interact both in vivo and in vitro,
Fig. 6. Specific binding of groES protein onto a groEL-bound Affi-Gel 10 matrix. Coupling of protein to Affi-Gel 10 matrix was carried out as described under "Materials and Methods." About 500 µg of groEL protein (or bovine serum albumin) and 0.6 ml of Affi-Gel 10 matrix were used for coupling. Fifty µl of [35S]methionine-labeled E. coli B178 extract (prepared as described under "Materials and Methods") were diluted to 200 µl with column buffer and loaded onto a 0.5-ml matrix at 4°C in a polypropylene Econo-Column. At the end of a 15-min incubation, the columns were washed with 4 × 1.0 ml each of column buffers containing 50 mM, 100 mM, and 2 M KC1 without ATP. Fractions were pooled, precipitated with 10% trichloroacetic acid, washed once with acetone, dried, resuspended in 50 µl of SDS-polyacrylamide gel sample buffer, and loaded onto a 12.5% SDS-polyacrylamide gel. The dried gels were exposed to Kodak SB5 film for 4 days at room temperature. A, represents the [35S]methionine-labeled polypeptides eluting at 200 mM KC1 from a bovine serum albumin-bound Affi-Gel 10 matrix; B, represents the [35S]methionine-labeled polypeptides eluting at 200 mM KC1 from a groEL-bound Affi-Gel 10 matrix. The arrow points to the position of the groES protein. Its identity was verified by two-dimensional gel electrophoresis with bona fide groES protein.

it is possible that the groES protein is also essential for the oligomerization of the B protein. This has not been directly demonstrated yet.

Mutations in either of the groE genes can result in a temperature-sensitive phenotype for bacterial growth (16, 18, 19), indicating that the groE proteins are essential, at least at high temperature. Recently, Wada and Itikawa (27) have shown that groE+ and groE− mutants exhibit a decreased rate of both RNA and DNA, but not protein syntheses, at high temperature. In this regard it is interesting that overproduction of the groES and groEL proteins has recently been shown to be both necessary and sufficient to suppress the DNA replication defect exhibited by some mutations in the dnaA initiator gene of E. coli (28).

Whatever the roles of groE proteins in E. coli DNA and RNA syntheses and in λ head assembly, many lines of evidence indicate that these proteins act at the same step, probably in association with one another. Now that the purified groE proteins are available, in vitro experiments, such as in vitro reconstitution of some of the λ assembly steps, can be designed to better understand their involvement in bacteriophage λ morphogenesis and also E. coli DNA macromolecular metabolism.

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REFERENCES