Escherichia coli cells that produce only plasmid-encoded wild-type or mutant GroEL were generated by bacteriophage P1 transduction. Effects of mutations that affect the allosteric properties of GroEL were characterized in vivo. Cells containing only GroEL(R197A), which has reduced intra-ring positive cooperativity and inter-ring negative cooperativity in ATP binding, grow poorly upon a temperature shift from 25 to 42 °C. This strain supports the growth of phages T4 and T5 but not phage λ and produces light at 28 °C when transformed with a second plasmid containing the lux operon. In contrast, cells containing only GroEL(R13G, A126V) which lacks negative cooperativity between rings but has intact intra-ring positive cooperativity grow normally and support phage growth but do not produce light at 28 °C. In vitro refolding of luciferase in the presence of this mutant is found to be less efficient compared with wild-type GroEL or other mutants tested. Our results show that allostery in GroEL is important in vivo in a manner that depends on the physiological conditions and is protein substrate specific.

In Vivo and in Vitro Function of GroEL Mutants with Impaired Allosteric Properties*  

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The Escherichia coli GroE system facilitates protein folding in vivo and in vitro in an ATP-dependent manner (for recent reviews see, for example, Refs. 1–4). It is composed of GroEL, an oligomer of 14 identical subunits that form two heptameric rings, stacked back-to-back, with 7-fold symmetry and a cavity at each end (5), and its helper-protein GroES which is a seven-membered ring of identical subunits (6). GroEL has 14 ATP-binding sites and a weak K⁺-dependent (7) ATPase activity. It undergoes ATP-induced conformational changes (8) that are reflected in binding of ATP with intra-ring positive cooperativity (9–11) and inter-ring negative cooperativity (12, 13).

Coupling between protein folding and allostery in the GroE system has recently been demonstrated in vitro (14). The importance of the allosteric properties of GroEL for its function in vivo remains, however, unclear. It has been questioned due to (i) the fact that the allosteric transitions of GroEL take place in vitro at subphysiological (micromolar) concentrations of ATP (13), and (ii) the finding that the apical domain of GroEL which is devoid of ATPase activity, is active in vivo (15). More recently, it has been demonstrated that the oligomeric structure of GroEL is required for biological activity because of the need for an intact cavity (16, 17). Evidence for the importance of the oligomeric structure of GroEL for activity in vivo owing to the requirement for proper allosteric communication within and between rings has, however, not been reported. We decided to begin addressing this issue by generating E. coli strains that express only plasmid-derived GroEL which is either wild type (as a control) or mutant with modified allosteric properties. The following GroEL mutants with different altered allosteric properties were chosen for analysis as follows: (i) GroEL(K4E) with disrupted inter-subunit contacts (18, 19); (ii) GroEL(R13G, A126V) with intact positive cooperativity and disrupted negative cooperativity (20); (iii) GroEL(R197A) with strongly diminished positive cooperativity and weakened negative cooperativity (12); (iv) GroEL(E409A, R501A) with increased positive and slightly weakened negative cooperativity (21), and (v) GroEL(R501A) with weakened positive cooperativity and disrupted negative cooperativity (21). To date, there have been very few studies on the in vivo consequences of mutations in GroEL known to modify its properties in vitro (see, for example, Ref. 22). Herein, we show that such mutations affect the function of GroEL in vivo (in cells that lack background chromosomal wild-type GroEL) and demonstrate that the effects depend on the physiological conditions and are protein substrate-specific.

**EXPERIMENTAL PROCEDURES**

**Materials**—Molecular biology reagents were purchased from Roche Molecular Biochemicals unless otherwise stated. The synthetic autoinducer N-(3-oxohexanoyl) homoserine lactone was kindly provided by A. Eberhard, Ithaca College, NY. All other reagents were obtained from Sigma or Aldrich. GroEL and GroES were purified as described (23).

**Subcloning**—The groEL gene was amplified from the pOAs plasmid (24) using Pwo DNA polymerase and the following oligonucleotides: FOR, 5′-GGG AAT TCA TCC GCG CAC GAC ACT G-3′; BACK, 5′-AAA ACG ACG GCC AGT G-3′. The FOR oligonucleotide is complementary to the region between GroEL and GroES except for the first 8 nucleotides. The BACK oligonucleotide is complementary to the region of the plasmid flanking the 3′ end of the gene. The reactions were carried out using Pwo DNA polymerase in 10 mM Tris-HCl buffer (pH 8.85 at 20 °C) containing 25 mM KCl, 5 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.8 mM of each dNTP, 140 pmol of each primer, and 0.5 μg of template DNA (containing the wild-type or mutant groEL gene). The PCR cycle consisted of 3 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 58 °C, and 2 min at 72 °C and, finally, 10 additional min at 72 °C. The 1.8-kilobase pair PCR products that contained the groEL gene were purified, digested by HindIII and EcoRI, heated for 5 min at 45 °C, repurified, and ligated overnight on ice to the plasmid pBAD30 (25) which was previously digested with the same enzymes. The groEL genes subcloned into the pBAD vector were fully sequenced. The pBAD vector contains the arabinose Pₐ₅ promoter which is induced by 1-ara-arabinose and repressed by glucose. This vector confers ampicillin resistance and has a pA-

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‡ The abbreviations used are: PCR, polymerase chain reaction; pfu, plaque-forming units.
in vivo function of GroEL mutants

CYC184 (P15A) origin of replication. It is designated pBADEL (mutant type).

A 12-kilobase pair SalI restriction fragment of the plasmid pBTK5 (26) containing the complete lux operon of Vibriobacteriophagedeletion (27) was cloned into pALTER-1 (Promega) previously digested with the same enzyme. This allows tetrazolium reduction and has the ColEl origin of replication that is compatible with the pBADEL vectors.

Creation of E. coli Strains with a Deletion of the Chromosomal groEL Gene—The chromosomal groEL gene of E. coli TG1 cells containing pBADEL (mutant type) was replaced with the nptII gene that confers kanamycin resistance by P1 transduction using A909/pBADEL as a donor (28). The A909/pBADEL strain is able to grow in the presence of L-arabinose but not encoded wild-type or mutant GroEL were tested for their ability to support the growth of phages λ(b2CI), T5, and T4(ΔD). The different TG1 ΔEL/pBADEL strains containing the lux operon of V. fischeri were grown overnight in 5 ml of LB medium containing 50 μg/ml ampicillin and then spun down in a microcentrifuge (6000 rpm for 10 min), resuspended in 5 ml of 0.1 M MgSO4 and 5 mM CaCl2, and gently shaken at 37 °C for 15 min. Following incubation for 20 min at 37 °C, 0.2 ml of 0.9 M trisodium citrate were added, and the cells were then spun down as above. The cells were gently resuspended in 1 ml of LB medium, shaken for 3 h at 37 °C, and then centrifuged (6000 rpm for 10 min). The supernatant was removed completely, and the cells were resuspended in 100 μl of LB medium and plated on LB plates containing 50 μg/ml kanamycin, 50 μg/ml ampicillin, and 0.2% l-arabinose.

Cell Growth—Overnight cultures grown at 25 °C were diluted 1:100 in 10 ml of LB medium containing 50 μg/ml ampicillin, 50 μg/ml kanamycin, and 0.02% arabinose in 100-ml flasks. The temperature was shifted to 42 °C after 2.5 h. Cell density was determined at different time intervals by measuring the optical density at 560 nm.

Phage Infection—TG1 ΔEL/pBADEL cells containing only plasmid-encoded wild-type or mutant GroEL were tested for their ability to support the growth of phages λ(b2CI), T5, and T4(ΔD). The different TG1 ΔEL/pBADEL strains containing the lux operon of V. fischeri were grown overnight in 10 ml of LB medium containing 50 μg/ml ampicillin, 50 μg/ml kanamycin, and 0.02% arabinose to an absorbance of 0.6 at 620 nm. One ml of each suspension was used to inoculate 100 ml of LB medium containing 50 μg/ml ampicillin, 50 μg/ml kanamycin, 0.2% l-arabinose, and 100 μl of stock phage. The cultures were then incubated at 37 °C in 250 ml of culture medium and monitored for plaque development for 24 h. Each plating was done in triplicate to ensure accuracy.

RESULTS

Effects of Mutations in GroEL on Bioluminescence—The different TG1 ΔEL/pBADEL strains containing the lux operon of V. fischeri were grown overnight with shaking at 30 °C in NZCYM broth containing 50 μg/ml kanamycin, 50 μg/ml ampicillin, 5 μg/ml tetracycline, and unless otherwise stated, 0.02% arabinose. The overnight cultures were then transferred to 20 or 28 °C, and growth was continued in the presence of 0.002% arabinose, the synthetic autoinducer N-(3-oxyhexanoyl) homoserine lactone. At 21 °C, the luminescence started to develop from the reactivation mixture was then immediately added followed by rapid addition of 300 μl of 0.005% (v/v) n-decyl aldehyde that had been freshly sonicated. The solution was vortexed for 5 s, and luminescence was measured using a Lumac 3M luminometer.

Cell Growth—The E. coli TG1 ΔEL/pBADEL cell strains containing only plasmid-encoded wild-type or mutant GroEL were tested for their ability to grow at different temperatures. No differences in the growth of the various strains were observed at 25 °C in the presence of 0.02 or 0.2% arabinose. Upon a temperature shift to 37 °C (data not shown) or 42 °C (Fig. 1), TG1 ΔEL/pBADEL(R197A) cells were found to reach a lower yield. The TG1 ΔEL/pBADEL strains expressing wild-type GroEL or mutants other than GroEL(R197A) were found to grow similarly.
found to propagate phages T4, T5, and λ by the different TG1ΔEL/pBADEL strains under different conditions.

| GroEL in cells Arabinose | T4  | T5  | λ   |
|--------------------------|-----|-----|-----|
| Wild-type 0.2            | +   | +   | +   |
| Wild-type 0.002          | +   | +   | +   |
| R197A 0.2                | +   | +   | −   |
| R197A 0.002              | +   | +   | −   |

Table 1: Propagation of phages T4, T5, and λ by the different TG1ΔEL/pBADEL strains under different conditions.

*Propagation or lack of propagation is indicated by + and −, respectively. Strains containing the other GroEL mutants in this study were found to propagate phages T4, T5, and λ like TG1ΔEL/pBADEL (wild type).*

incubated for 20 h at 30 °C. Upon transfer to 28 °C, luminescence started to develop with kinetics and intensity which were found to be GroEL mutant-specific. After 2 h at 28 °C, all the strains produced light, but the amount of light produced by the TG1ΔEL/pBADEL strains expressing GroEL(R501A) and GroEL(R13G, A126V) was found to be significantly lower (Fig. 3, left). It may be seen in Fig. 3 that the amount of light produced is not necessarily proportional to the cell density. TG1ΔEL/pBADEL(R501A), for example, grew well but produced little light, whereas TG1ΔEL/pBADEL(R197A) grew poorly but produced much more light (Fig. 3, right). In order to investigate this issue further, we followed the development of luminescence by each of the TG1ΔEL/pBADEL strains at different temperatures and initial cell densities (Fig. 4). All the strains grew similarly during the course of the experiments (not shown) and produced light at 20 °C at a high cell density (Fig. 4B). The maximal light production of strains containing either GroEL(R501A) or GroEL(R13G, A126V) was found to be less than that of the other strains in agreement with results shown in Fig. 3. These two strains and also the strain containing GroEL(E409A, R501A) did not produce any measurable light at 28 °C, whereas the strains containing GroEL(wild-type), GroEL(K4E), or GroEL(R197A) produced light at 28 °C but less than at 20 °C. The TG1ΔEL/pBADEL(R197A) strain produced light at 28 °C at a low cell density, whereas at high cell densities it did not produce any light (Fig. 4, C and D) in agreement with the results shown in Fig. 3. The strains containing GroEL(wild-type), GroEL(K4E), or GroEL(R197A) produce more light at 20 °C than the three strains containing either GroEL(R501A), GroEL(R13G, A126V), or GroEL(E409A, R501A) which at 28 °C do not produce any light. All the strains were found to produce light at 28 °C if the synthetic autoinducer was added, but the light production by the TG1ΔEL/pBADEL(R501A) and TG1ΔEL/pBADEL(R13G, A126V) strains was very low (data not shown).

Effects of Expression Level of GroEL on Luminescence—The development of luminescence by the TG1ΔEL/pBADEL strains containing wild-type GroEL, GroEL(R197A), and GroEL(R501A) was followed at 28 °C in the presence of different concentrations of arabinose, which induces expression of GroEL (25). In the presence of 0.01% arabinose, the TG1ΔEL/pBADEL(wild-type) and TG1ΔEL/pBADEL(R197A) strains produced light, whereas the TG1ΔEL/pBADEL(R501A) strain did not (not shown). In the presence of 0.2% arabinose, only the TG1ΔEL/pBADEL(wild-type) strain was found to produce light (Fig. 4D). In the presence of 0.2% arabinose none of these three strains produced light (not shown).

Effects of Mutations in GroEL on Reactivation of Denatured V. fischeri Luciferase α and β Subunits—Luciferase α and β subunits that had been denatured in 6 M urea and 10 mM HCl were found to fold spontaneously to a small extent upon dilution into folding buffer (Fig. 5A). In the presence of wild-type GroEL (Fig. 5A) or the different mutants, folding was completely arrested indicating that denatured α and/or β luciferase subunits bind tightly to GroEL. Reactivation was found to require the full GroE system and was arrested also in the presence of GroEL and ATP except in the case of the GroEL(R13G, A126V) mutant (Fig. 5B). In the presence of wild-type GroEL, GroES, and ATP, the yield of reactivation was increased about 4-fold relative to spontaneous folding (Fig. 5A). The yield of reactivation by GroEL(R13G, A126V), in the presence of GroES and ATP, was about 50% that of wild-type GroEL (Fig. 5C). The yields of reactivation by the other mutants in the presence of GroES and ATP were found to be similar to that of wild-type GroEL.

**DISCUSSION**

Bacteriophage P1 transduction was used to generate E. coli TG1 strains that express only plasmid-derived wild-type GroEL or mutants with various modified and well-characterized allosteric properties. The effects of these mutations on the function of GroEL in vivo were studied using the following two assays: (i) propagation of phages λ, T4, and T5 and (ii) the bioluminescence of cells containing the full lux operon of V. fischeri. The GroE system was first identified by genetic studies of bacteriophage growth (30). Bacteriophages λ (31, 32) and T5 (33) employ the host GroE system for the folding of their own proteins. Bacteriophage T4 uses host GroEL but its own co-chaperonin, Gp31, for the folding of its major capsid protein, Gp23 (34, 35). Luminescence in V. fischeri ceil s and in E. coli cells that contain the lux genes requires the product of the luxR gene which activates transcription of the lux operon upon binding to an autoinducer (27). The GroE system is believed to facilitate the folding in vivo of the LuxR protein (36, 37) and possibly also the luciferase α (LuxA) and β (LuxB) subunits (38). The lux operon also contains luxC, luxD, and luxE which code for enzymes required for synthesis of the long chain aldehyde luciferase substrate, luxI which codes for an enzyme involved in autoinducer synthesis and luxG which codes for a...
FIG. 3. Luminescence at 28 °C of TG1ΔEL/pBADEL cells containing the lux operon of V. fischeri. The different TG1ΔEL/pBADEL strains were streaked on a NZCYM plate containing the appropriate antibiotics and 0.02% arabinose. The plate was incubated at 30 °C for 20 h and then transferred to 28 °C for 1.5 h before being photographed using a Nikon model TEA CCD-512 TKB/1 camera and a 1-s exposure in the dark (left) or in daylight (right).

FIG. 4. Effects of temperature and cell density on the kinetics of onset of luminescence in TG1ΔEL/pBADEL cells containing the lux operon of V. fischeri. Cultures were inoculated, diluted in a 2-fold serial manner, and grown overnight with shaking at 30 °C in NZCYM broth containing the appropriate antibiotics and 0.02% arabinose. Overnight cultures that reached an optical density at 600 nm of 0.3 (±0.1) (A and C) or 1.5 (±0.2) (B and D) were transferred to 20 °C (A and B) or 28 °C (C and D), and shaking was continued. At different times, 100 µl of the cultures were removed, and their luminescence was measured using a Microtox model 2055 luminometer.
protein with unknown function (27).

The functional consequences in vivo of mutations that alter the allosteric properties of GroEL are found in this study to be protein substrate-specific. Cells containing GroEL(R197A), for example, grow poorly at 37 °C (not shown) and 42 °C (Fig. 1) and do not support growth of phage λ but do support growth of phages T4 and T5 (Fig. 2) and the folding of the lux operon gene products and other proteins that may be required for light production (Figs. 3 and 4). Cells containing GroEL(R501A) or GroEL(R13G, A126V), on the other hand, support the growth of phages λ, T4, and T5 (not shown) but not the folding at 28 °C of one or more of the proteins required for bioluminescence (Figs. 3 and 4). The need for specific allosteric properties in GroEL therefore depends on the nature of the protein substrates.

The requirement for the GroE system for folding in vivo can be circumvented by changing conditions in a substrate-specific manner. All the TG1ΔEL/pBADEL strains examined in this study produce light at 20 °C (Fig. 4B), thus suggesting that unassisted folding of the proteins required for bioluminescence is more efficient at this temperature as, for example, observed in the case of ribulose-bisphosphate carboxylase/oxygenase (7). All the TG1ΔEL/pBADEL strains examined in this study also produce light at 28 °C when inducer is added (not shown), perhaps because the active conformation of LuxR is stabilized in its presence (27). Light production by cells containing GroEL(R501A) or GroEL(R13G, A126V) at 28 °C is, however, very low in the presence of inducer. Interestingly, the TG1ΔEL/pBADEL(R197A) strain which grows poorly at 37 and 42 °C is the only one that produces relatively more light at a low cell density, perhaps because folding of proteins which inhibit luminescence, such as LexA (27), is not facilitated by GroEL(R197A).

The effects in vivo of mutations in GroEL are also found to depend on its level of expression. If the GroEL mutant has low affinity for unfolded protein substrates then increasing its concentration may reduce the effect of the mutation in vivo. This may explain why the TG1ΔEL/pBADEL(R197A) strain is not viable at 37 °C when the concentration of arabinose is less than 0.002% (Fig. 2). If, however, the GroEL mutant binds unfolded proteins but does not release them (i.e. it is a “trap” mutant), then lowering its concentration may diminish the effect of the mutation. For example, all the TG1ΔEL/pBADEL strains produce less light at 28 °C in the presence of 0.2% arabinose than in the presence of 0.02% arabinose (not shown). Changes in the expression level of GroEL also alter the GroEL/GroES ratio in the cell which, although physiologically important, does not affect the conclusions in this study since the amount of GroEL in the different strains was determined to be the same (not shown).

An understanding of how the mutations in GroEL affect its function in vivo requires identification of the relevant substrate protein(s) whose misfolding leads to the observed phenotype and establishing the mechanism by which the mutation causes the misfolding. Here, we concentrated on trying to understand the reasons for differences in bioluminescence of the different TG1ΔEL/pBADEL strains containing the lux operon. We initially focused on LuxR as the substrate of GroEL because of reports in the literature that GroE facilitates the folding in vivo of the LuxR protein (36, 37). GroEL was found to bind denatured LuxR and release it in an ATP- and GroES-dependent manner, but no differences in binding or release of LuxR by the different mutants were observed (not shown) in agreement with the small effect of the autoinducer on light production by the TG1ΔEL/pBADEL(R501A) and TG1ΔEL/pBADEL(R13G, A126V) strains. Next we analyzed GroE-assisted reactivation of denatured α and β luciferase subunits. The GroE system was previously shown to facilitate the in vitro folding of bacterial luciferase from Vibrio harveyi (38). The extent of reactivation of α and β luciferase subunits by wild-type GroEL and the various mutants, in the presence of GroES and ATP, was found to be similar except in the case of GroEL(R13G, A126V) where the yield was about 50% that of the others (Fig. 5C). GroEL(R13G, A126V) lacks negative cooperativity between rings but has intact intra-ring positive cooperativity and a $k_{cat}$ of ATP hydrolysis similar to that of wild-type GroEL (20). This mutant was also found to differ from wild-type GroEL and the other mutants in being able to release bound luciferase in the presence of ATP alone (Fig. 5B). It was recently shown that the ATP-bound conformation of GroEL(A126V) is similar to the GroES-bound conformation of wild-type GroEL (39) thus explaining why ATP by itself can trigger release of substrates.
bound to GroEL(R13G, A126V). ATP-triggered release of luciferase subunits in a conformation not yet committed to fold may contribute to the lower yield of the presence of GroEL(R13G, A126V) relative to wild-type GroEL. The GroEL(A126V) mutant was also found to form symmetric 2:1 GroES-GroEL complexes (39) in which substrate-binding sites are blocked. These properties of GroEL(A126V) may explain why cells containing GroEL(R13G, A126V) do not produce light at 28 °C. We do not yet know which proteins required for bioluminescence fail to fold in the presence of GroEL(R501A) and also which proteins involved in cell growth and λ phage propagation fail to fold in the presence of GroEL(R197A) at 25 and 37 °C.

Our results suggest that mutations that perturb specific steps in the reaction cycle of GroEL are likely to be relatively more damaging. For example, cells containing only GroEL(D398A), which is defective in ATP hydrolysis, exhibit a normal phenotype. This phenotype may be due, in part, to assembly into oligomeric structures in the presence of physiological concentrations of ATP (data not shown). GroEL(K4E) and also which proteins involved in cell growth and λ phage propagation fail to fold in the presence of GroEL(R197A) at 25 and 37 °C.

In summary, our results indicate that allosteric communication in GroEL is important for the in vivo folding of a subset of substrates under certain physiological conditions. The poor growth of TG1ΔEL/pBEDEL(R197A) at 42 °C and the formation of inclusion bodies in these cells (not shown) suggests that intact positive cooperativity may be of particular importance under stress conditions. Disrupted positive cooperativity reduces the shift in equilibrium under stress conditions toward the protein acceptor state of GroEL. HPrtL and co-workers (40) recently identified a set of in vivo substrates of GroEL. Our results suggest that a “universal” set of protein substrates does not exist and that the set of substrates that interact with GroEL in vivo depends on the physiological conditions.

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