A Mutation in GroEL Interferes with Protein Folding by Reducing the Rate of Discharge of Sequestered Polypeptides*

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GroEL140, a mutant Escherichia coli chaperonin unable to support bacteriophage λ head assembly, was purified to near homogeneity and compared to wild type GroEL (cpn60). GroEL140 exhibited a 1.5-fold lower ATPase activity relative to the wild type protein. The hydrolysis of ATP by both polypeptides was fully inhibited by an excess of ATPγS and partially inhibited by ADP and 5'-adenylimidodiphosphate, suggesting that adenine nucleotides display different affinities for the ATP binding site of chaperonins. GroEL140 was more sensitive to trypsin digestion compared to wild type GroEL indicating that the mutation destabilized the conformation of the mutant. The proteolytic susceptibility of both chaperonins was similarly enhanced upon addition of ATP, ADP or non-hydrolyzable ATP analogs, providing evidence (i) of a conformational change in the chaperonin structure which is likely to drive the protein discharge process, and (ii) that hydrolysis of ATP is not required to achieve topological modifications. GroEL140 retained its ability to bind non-native ribulose bisphosphate carboxylase/oxygenase (Rbu-Pz-carboxylase), but released bound proteins upon addition of ATP and GroES (cpn10) 6–7-fold less efficiently compared to GroEL. This functional defect was shown to be related to a suboptimal, but not an absence of, interaction with GroES since (i) GroEL140 and GroES were unable to form a complex isolatable by size exclusion chromatography, and (ii) increasing the incubation time or the concentration of GroES enhanced the amount of refolded Rbu-Pz-carboxylase discharged from GroEL140-Rbu-Pz-carboxylase binary complexes. Pulse-chase experiments involving a double immunoabsorption technique confirmed that Rbu-Pz-carboxylase remained associated two times longer with GroEL140 than with GroEL in vivo.

Protein folding has recently emerged as a central issue in both fundamental and applied research. The isolation and characterization of molecular chaperones which can modulate the folding and subsequent oligomerization, transport, and stability of polypeptides (reviewed in Refs. 1–5) has certainly contributed to a renewed interest in the pathways proteins follow to reach their final conformation. Molecular chaperones have been conserved throughout evolution and have been identified in most of the organisms and cellular compartment studied.

One of the most abundant molecular chaperones in bacteria is chaperonin GroEL (also known as cpn60 or hsp60 in different organisms), a molecule composed of 14 identical subunits (M, ~ 57,000) which associate to form a double stack of heptameric rings in prokaryotic and plant cells (2, 6). Recent investigations have, however, shown that the mammalian mitochondrial homolog of GroEL can exist as a functional single heptameric ring (7). GroEL, which is essential for cell survival (8), was initially identified for its requirement in the assembly of λ and T4 phage heads, and that of T5 tails (9). Genetic evidence also suggests that GroEL is involved in DNA replication (10, 11), cell division (12), and protein transport (13–15). GroEL displays a weak ATPase activity which is dependent upon the presence of magnesium and potassium ions (16), and promotes its own assembly (17). Compelling genetic (14, 15, 18–20) and biochemical (16, 21, 22) evidence indicates that GroEL functionally interacts with the heptameric cochaperonin GroES (also known as cpn10).

GroEL and related chaperonins associate with non-native polypeptides, thereby preventing the proteins from entering unproductive aggregation pathways, and are able to discharge bound proteins in a biologically active conformation upon addition of ATP and GroES (16, 21, 23–27). However, many of the mechanistic details of this process remain obscure. In this paper, we have purified a mutant GroEL protein and compared it to the wild type in an effort to gain some insight on the mechanism of action of chaperonins. The mutant GroEL140 was found to qualitatively behave as the wild type protein in its ability to bind non-native proteins, to promote ATP hydrolysis, and in its susceptibility to proteolysis. Nevertheless, it was less efficient and more sensitive to proteolysis than wild type GroEL. Furthermore, the mutant chaperonin was inefficient in discharging bound proteins in a biologically active form. We determined that the decreased yields were related to an impaired ability of GroEL140 to bind GroES. The implications of these experiments for the mechanism of action of chaperonins are discussed.

MATERIALS AND METHODS

Strains and Plasmids—Strains B178 (W3110 F- galE relA), CG714 (B178 groEL140) (28), and plasmid pRR2119 (29) have been described previously.

GroEL140 Purification—Two shake flasks containing 1 liter of LB medium (Difco) supplemented with 0.2% glucose were inoculated at a 1:20 dilution with an overnight inoculum of CG714 cells grown in LB medium at 30 °C. Cultures were incubated with agitation at 30 °C to late exponential phase (OD600 = 0.8) and rapidly shifted to 42 °C by transfer to a 60 °C water bath. Incubation was continued at 43 °C for an additional 50 min, at which point the cells were pelleted by centrifugation at 10,000 rpm × 10 min. The pellet was resuspended in 20 ml of buffer I (50 mM Tris-HCl, pH 7.2, 0.1 mM EDTA, 1 mM

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DTT) and disrupted by four successive cycles of French pressing at 20,000 psi. Cellular debris were removed by centrifugation at 12,000 rpm x 30 min and the clarified lysate was filtered through a 0.22-μm Amicon filter. Proteins were loaded on a 16 x 5.2-cm Amicon column packed with Bio-gel A-5m (Pharmacia LKB Biotechnology) and equilibrated with buffer I.

The column was developed at 2 ml/min with a 525-ml gradient from 0 to 1 M NaCl in buffer I at 4 °C. Fractions eluting between 55 and 62% NaCl were pooled, concentrated by ultrafiltration using a YM-30 membrane (Amicon), and loaded on a Superose 6-10/30 column (Pharmacia) developed in buffer II (100 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 10 mM KCl, 0.01% Tween 20) at 0.5 ml/min using the Pharmacia fast protein liquid chromatography system. The fractions eluting between 12 and 13 ml (the column void volume is 10 ml) were pooled, concentrated using Amicon FM-10 microconcentrators, and rechromatographed on Superose 6. The GroEL140 peak fractions were concentrated, supplemented with 10% glycerol, frozen in liquid nitrogen, and stored at −80 °C. The protein concentration was 45 μM (protomer) as estimated by amino acid analysis and video scanning. GroEL140 appeared about 95% pure on overloaded SDS gels and migrated at the same position as wild type GroEL on 6% non-denaturing gels both in the presence and absence of Mg-ATP.

Proteolytic Digestion—All proteolytic digestion experiments were performed in buffer II at 37 °C using a 0.17 mg/ml solution of trypsin (Sigma) in a total reaction volume of 100 μl. Briefly, 8 μl of GroEL (64 μM protomer) or GroEL140 (45 μM protomer) were mixed in buffer II with 1 ml of Trypsin (10 μM) and the samples were incubated at 0 °C. For some experiments 3.2 μl of purified GroES (308 μM protomer) was added to the reaction mixture. This GroES concentration corresponded to a 3.8 100% value.

The amount of protein present in the GroEL band was estimated by absorbance at 280 nm and by amino acid analysis. GroEL140 appeared about 95% pure on overloaded SDS gels and migrated at the same position as wild type GroEL on 6% non-denaturing gels both in the presence and absence of Mg-ATP.

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Extraction and assay medium (Difco), 50 μg/ml ampicillin to an OD, 0.2. Rbu-P₂-carboxylase was acid denatured by 312.5-fold dilution in 10 mM HCl (final concentration 10 μM) followed by incubation at room temperature for 30 min. The refolding buffer (buffer IV) consisted of 42 mM Tris-HCl, pH 7.7, 8.4 mM MgCl₂, 11.7 mM KCl, 100 mM bovine serum albumin (BSA), 3.2 μl of purified GroES (308 μM protomer) was added to the reaction mixture. This GroES concentration corresponded to a 3.8 100% value.

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VII containing 0.2 M NaCl, and the immunoabsorbed binary complexes between GroEL or GroEL140 and substrate proteins were disrupted and released by two 100-ml washes of 1% SDS at 90 °C. The SDS samples were pooled, diluted with 1 ml of buffer VIII (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100), and incubated with anti-GroEL and anti-Rbu-P2-carboxylase to identify the two radiolabeled proteins of interest. After subsequent absorption to Protein A-Sepharose and washing (31), the proteins were identified by SDS-PAGE and fluorography using radiolabeled GroEL and Rbu-P2-carboxylase as markers. GroEL and Rbu-P2-carboxylase bands were excised from dried gels, incubated with NCS tissue solubilizer (Amersham Corp.) at 50 °C overnight, neutralized with acetic acid and counted in Econofluor 2 (Du Pont-New England Nuclear).

RESULTS

Purification of GroEL140—Escherichia coli strain CG714 encodes a mutant GroEL protein and was initially isolated for its inability to support bacteriophage λ head morphogenesis (28). The mutation in GroEL140 was recently mapped. It corresponds to the single amino acid substitution Ser201 → Phe201. Transcription of the groE operon, including the mutant groEL140 gene, is under the control of the Eo32 and Er32 promoters (9). Neidhardt et al. (32) have shown that a temperature shift from 37 to 46 °C increases the synthesis of native GroEL from 2 to 10% of the total cellular protein, primarily because of enhanced expression by the Er32 promoter (9). Therefore, to maximize groEL140 expression, CG714 was grown to late exponential phase in LB medium at 30 °C, rapidly shifted to 42 °C, and incubated for 50 min at this temperature (see “Materials and Methods”). GroEL140 was isolated from disrupted cells by DEAE-Sephadex ion-exchange chromatography followed by two gel filtration steps. The concentrated GroEL140 protein migrated as wild type GroEL on non-denaturing SDS gels and was estimated to be more than 95% pure by video scanning of overloaded SDS gels (data not shown).

ATPase Activity and Inhibition—Bacterial GroEL displays a weak ATPase activity which fully depends on the presence of magnesium and potassium ions (16). Since the binding and hydrolysis of ATP appear to play a major role in the release of folding intermediates associated with several molecular chaperones, we first determined the influence of single amino acid substitution in GroEL140 on the ATPase activity of this protein. Under the experimental conditions chosen (see “Materials and Methods”), the ATP turnover was 1.45 × 10⁻² μmol of ATP/μmol of GroEL140 (protomer)/s with a standard deviation of 0.011 × 10⁻². This result was about 1.5-fold lower compared to the turnover number obtained with wild type GroEL (2.11 × 10⁻² ± 0.073 × 10⁻², Fig. 1). When potassium ions were omitted, no ATPase activity could be detected with either GroEL or GroEL140. The ATPase activity, however, was fully restored upon addition of 100 mM KCl to the reaction mixture (arrow in Fig. 1). This result indicated that neither purified preparations were contaminated with an extraneous, potassium independent, ATPase activity.

ATP analogs have been reported to influence the release of folding intermediates complexed with GroEL (24, 26, 27). To verify that these nucleotides could indeed interact with the ATP-binding site of cpn60, the above experiment was repeated in the presence of 1 mM AMP-PNP, ATPγS, or ADP. Table I shows that all adenine nucleotides examined were able to inhibit the ATPase activity of both GroEL and GroEL140, presumably by competing for the ATP-binding site. ATPγS fully inhibited hydrolysis in both the wild type and the mutant protein, suggesting that this ATP analog could efficiently recognize the ATP-binding site on both the wild type and mutant chaperonins. ADP was slightly less effective and conferred 80 and 70% inhibition in GroEL and GroEL140, respectively. AMP-PNP inhibition was inefficient with GroEL140 but still appreciable with wild type GroEL (Table I). The above result suggests a poor binding of AMP-PNP to GroEL140 possibly due to modifications in the conformation of the mutant protein.

Proteolytic Digestion—Conformational changes have been proposed to play a major role in the binding of folding intermediates and in the discharge of polypeptides from molecular chaperones. However, very little information is currently available on the various conformational states of GroEL. We have used protease digestion to determine the extent of topological modifications resulting from the binding and hydrolysis of ATP by GroEL. Fig. 2 shows that although native GroEL is resistant to trypsin digestion under the experimental conditions chosen (panel A, see “Materials and Methods”), about 50% of GroEL140 was degraded following 30 min of incubation with trypsin at 37 °C (panel B). Addition of 2.5 mM ATP to the reaction mixture significantly enhanced the proteolytic susceptibility of both GroEL and GroEL140, reducing their half-lives to 22 and 3.6 min, respectively. Since both the binding and hydrolysis of ATP are likely candidates for inducing a structural change, the experiment was repeated in the presence of 5 mM ADP or 5 mM of the non-hydrolyzable ATP analogs AMP-PNP and ATPγS. A comparable, or in some instances an enhanced, susceptibility to proteolysis was observed when the adenine nucleotides were incubated with GroEL or GroEL140. These results suggest that the binding, and not the hydrolysis of ATP, was responsible for exposing more arginine and lysine residues. The fact that GroEL140 is consistently more susceptible to trypsin digestion relative to the wild type can be interpreted as a more loosely assembled conformation directly resulting from the single amino acid substitution in this protein.

In contrast, the addition of a 3.8-fold molar excess of (GroES)2 over (cpn60), had essentially no effect on the ATP-dependent degradation of GroEL or GroEL140 by trypsin (Fig. 2, panel C). It is interesting to note that in the presence of adenine nucleotides, the degradation of GroEL and GroEL140 by trypsin apparently occurs in a two-step process consisting of a rapid hydrolysis phase followed by a slower degradation stage. The first phase is completed within 5 min with GroEL140 and about 10 min with native GroEL. However, the rate of degradation of both proteins in the second,

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2 C. Georgopoulos, personal communication.
3 F. Baneyx, unpublished results.
TABLE I
Inhibition of the ATPase activities of GroEL and GroEL140 by adenine nucleotides

| Nucleotides | GroEL ATPase activity | % inhibition | GroEL140 ATPase activity | % inhibition |
|------------|-----------------------|--------------|--------------------------|--------------|
| ATP        | $2.10 \times 10^{-2}$  | 0            | $1.45 \times 10^{-2}$  | 0            |
| ATP + AMP-PNP | $0.81 \times 10^{-2}$  | 61           | $0.91 \times 10^{-2}$  | 37           |
| ATP + ADP  | $0.41 \times 10^{-2}$  | 80           | $0.42 \times 10^{-2}$  | 71           |
| ATP + ATPγS | $<10^{-3}$             | 100          | $<10^{-3}$               | 100          |

FIG. 2. Differential susceptibility of GroEL and GroEL140 to trypsin digestion at 37°C: influence of nucleotides and GroES. GroEL (panel A) or GroEL140 (panel B) were incubated with trypsin with no additives (O), 2.5 mM ATP (A), 5 mM ADP (B), 5 mM AMP-PNP (C), or 5 mM ATPγS (D). Panel C is a comparison of the degradation profiles of GroEL + 2.5 mM ATP (O), GroEL + 2.5 mM ATP + GroES (A), GroEL140 + 2.5 mM ATP (A), and GroEL140 + 2.5 mM ATP + GroES (A). Conditions and concentrations are described under "Materials and Methods.

The slower phase appears to be almost identical.

Binding and Release of Rbu-P2-carboxylase to GroEL and GroEL140—Rhodospirillum rubrum Rbu-P2-carboxylase consists of two identical monomers (L1) which associate to yield a biologically active enzyme (L2) (33). Non-native Rbu-P2-carboxylase tightly interacts with GroEL but can be mostly released in an active form when GroES, Mg-ATP, and K+ ions are present (16, 21). The extent of Rbu-P2-carboxylase refolding can be readily determined in an enzyme activity assay which measures the incorporation of 14C into acid stable products (34). In addition, the physical existence of the discharged L2 enzyme was recently demonstrated by size exclusion chromatography (30). Therefore, we decided to use R. rubrum Rbu-P2-carboxylase as a substrate to study the interaction of non-native polypeptides with GroEL140. Fig. 3 shows that acid-denatured [35S]Rbu-P2-carboxylase forms a stable binary complex with GroEL140 eluting in the void volume of the TSK3000 size exclusion column. About 70% of the total radioactivity was present in the GroEL140 peak (compared to about 80% for wild type GroEL). This implied that the mutation in GroEL140 did not significantly reduce the affinity of non-native polypeptides for GroEL140. However, the mutant appeared to be compromised in its ability to discharge Rbu-P2-carboxylase. Addition of 2.5 mM ATP to the binary complex released over 75% of the radioactivity associated with GroEL but only 18% of that complexed with GroEL140 (Table II). In both cases, however, most of the released material did not fold in a monomeric or dimeric form and remained trapped in the column (Table II). This result was previously observed (30) and indicates that the mere presence of ATP weakens the affinity of GroEL for Rbu-P2-carboxylase. When both ATP and GroES were added, a significant increase in Rbu-P2-carboxylase release was observed, and two peaks corresponding to the monomeric and dimeric forms of Rbu-P2-carboxylase were detected (Fig. 3, Ref. 30). Under these conditions, more than 50% of the protein loaded onto GroEL140 was discharged compared to over 90% for GroEL (Table II). Furthermore, while 82% of the material dissociated from GroEL was recovered either in dimeric (62%) or monomeric form (20%), only 48% of the Rbu-P2-carboxylase released from GroEL140 was obtained in a dimeric (28%) or monomeric form (22%).

FIG. 3. Non-native Rbu-P2-carboxylase loaded onto GroEL140 is released from the chaperonin in the presence of both ATP and GroES. Acid unfolded [35S]Rbu-P2-carboxylase was loaded on GroEL140 and incubated without additives (O), 2.5 mM ATP (C), or 2.5 mM ATP and an excess of GroES (A). The complex was separated by size exclusion chromatography on a TSK3000 column as described under "Materials and Methods." The position of the Rbu-P2-carboxylase monomer (L1) and the biologically active dimer (L2) are indicated by arrows.
In Vitro Interaction between GroEL140 and GroES—Since the low efficiency of Rbu-P2-carboxylase refolding by GroEL140 could only be marginally attributed to its reduced ATPase activity, and was not related to a poor binding of folding intermediates to the mutant chaperonin, we investigated the interaction of the cochaperonin GroES with the mutant GroEL140. In the presence of ATP, GroES forms a stable complex with GroEL at a 1 to 1 ratio (7-mer and 14-mer, respectively; Ref. 16). The dissociation constant for the complex is relatively low since it can be isolated by size exclusion chromatography (Ref. 16; Fig. 4, lane 2). GroEL140, incubated with a 5.6-fold excess of GroES, in the presence of 2.5 mM ATP and resolved on a Superose 6 column equilibrated with 0.25 mM ATP as described under “Materials and Methods.” The fractions corresponding to the GroEL140 peak were acetone precipitated and visualized by Western blotting using anti-GroES as a primary antibody (see “Materials and Methods”). The experiment was repeated with wild type GroEL as a control. Fig. 4 shows that GroES could not be detected in the GroEL140 peak (lane 3), while a band migrating at the same position as purified GroES was evident in the fractions isolated from the GroEL peak (10 kDa arrow, lanes 1 and 2). Since the anti-GroES antiserum is slightly cross-reactive with GroEL, faint bands corresponding to the GroEL and GroEL140 proteins were also visible on the blot (Fig. 4, 60 kDa arrow). Our inability to isolate a complex between GroES and GroEL140 could be explained by (i) a total absence of binding between GroES and the mutant chaperonin, or (ii) a weaker interaction between these proteins resulting in a larger dissociation constant. Nevertheless, since GroEL140 was able to yield at least some dimeric (L1) Rbu-P2-carboxylase (Table II) and because both GroES and ATP are required to achieve optimal results (Table II, Ref. 21), we suspected that the two proteins could still interact, albeit suboptimally. This hypothesis was tested as follows.

![Fig. 4. GroEL140 is unable to form a complex with GroES](image)

**TABLE II**

Release of [35S]Rbu-P2-carboxylase loaded onto GroEL or GroEL140 by ATP or ATP and GroES

| Additive | % release from GroEL | % radioactivity in L1 peak | % radioactivity in L2 peak | % release from GroEL140 | % radioactivity in L1 peak | % radioactivity in L2 peak |
|----------|----------------------|---------------------------|---------------------------|-------------------------|---------------------------|---------------------------|
| None     | 0                    | <4.0                      | <4.0                      | 0                       | <3.0                      | <3.0                      |
| ATP      | 77.3                 | 6.4                       | 8.2                       | 18.2                    | 3.0                       | 11.5                      |
| ATP + GroES | 91.5              | 57.3                      | 18.2                      | 52.7                    | 13.9                      | 11.5                      |

Acid-denatured [35S]Rbu-P2-carboxylase was loaded onto GroEL or GroEL140 and incubated with no additives, 2.5 mM ATP, or 2.5 mM ATP and a 7-fold molar excess of GroES, as described under “Materials and Methods.” Results are expressed as percent of the radioactivity associated with the GroEL/GroEL140 peak.

Rbu-P2-carboxylase unfolded by incubation in 10 mM HCl was loaded onto an excess of GroEL140 or GroEL as a control, and supplemented with a 3-fold molar excess of GroES (7-mer over 14-mer) and 9 mM ATP. The reaction mixture was incubated at room temperature for increasing periods of time and the amount of refolded Rbu-P2-carboxylase estimated periodically. Fig. 5A shows that Rbu-P2-carboxylase refolding by wild type GroEL was essentially complete after 3 h of incubation at room temperature. GroEL140 was much less efficient in achieving refolding. After 90 min of incubation, the Rbu-P2-carboxylase activity for the GroEL140 sample was only 15% of that obtained with the wild type GroEL sample. Although the amount of active Rbu-P2-carboxylase refolded by GroEL140 steadily increased with time, it was

**FIG. 5. Inefficient release of Rbu-P2-carboxylase by GroEL140 can be improved by increasing the incubation time or the GroES concentration.** Panel A, effect of increased incubation time. Acid unfolded Rbu-P2-carboxylase was loaded onto GroEL (⊗) or GroEL140 (△) and incubated for increasing amounts of time in the presence of ATP and GroES. The amount of refolded Rbu-P2-carboxylase was determined as described under “Materials and Methods.” Incomplete reactions mixtures of Rbu-P2-carboxylase loaded onto GroEL (■) or GroEL140 (▲) were supplemented with ATP (black arrow) and GroES (white arrow) after 90.5 and 270.5 min of incubation respectively. Panel B, Rbu-P2-carboxylase loaded onto GroEL140 was incubated in the presence of ATP and an increasing molar excess of GroES. The Rbu-P2-carboxylase refolded was assayed after 90 min of incubation at room temperature.
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In a separate experiment, we tested the influence of increasing concentrations of GroES on the GroEL140-mediated folding of Rbu-P2-carboxylase. Fig. 5 B shows that as the molar excess of (GroES) over (GroEL140) increased, more Rbu-P2-carboxylase could be discharged from the mutant chaperonin. Interestingly, the same amount of active Rbu-P2-carboxylase (15% of the activity of the native Rbu-P2-carboxylase control) was obtained by either quadrupling the incubation time or quadrupling the excess of GroES. Thus, the above results demonstrate that GroEL140 is still able to interact with GroES and discharge biologically active Rbu-P2-carboxylase although the yield of the process is between 6-7-fold lower compared to native GroEL.

**DISCUSSION**

In spite of the growing body of information dealing with molecular chaperones, very little is known about the in vivo function and mechanism of action of these proteins. The *E. coli* chaperone DnaK, its mammalian homolog hsp72/73, and the related endoplasmic reticulum protein BiP, have been extensively studied, and some clues regarding their mode of action have been recently obtained. Specific conformational changes in the molecule resulting from the binding and hydrolysis of ATP have been implicated in the refolding process. However, their role in vivo is still unclear. For GroEL, as would be anticipated if Rbu-P2-carboxylase has a longer occupancy time on the mutant GroEL protein. The increased time of interaction between the mutant chaperonin and non-native proteins may explain the slower growth characteristics of CG714 compared to the parental strain B178.

![Fig. 6. Chaperonin-Rbu-P2-carboxylase binary complexes formed in vivo are more stable with GroEL140 than with wild type GroEL.](Image)

**Table:**

| Time (min) | GroEL140 | GroEL |
|-----------|----------|-------|
| 0         | 100%     | 100%  |
| 1         | 70%      | 50%   |
| 4         | 30%      | 20%   |

**Panel A:** GroEL-Rubisco bands at different time points and normalizing the result to the radioactivity in the GroEL bands at the same times. Fig. 6 shows that Rbu-P2-carboxylase remained associated with GroEL140 about twice as long as with wild type GroEL, suggesting that the process of polypeptide release was also less efficient in vivo. Also note that immediately following the 2-min pulse (panel A, 0 min chase), there was significantly more Rbu-P2-carboxylase associated with GroEL140 compared with GroEL, as would be anticipated if Rbu-P2-carboxylase has a longer occupancy time on the mutant GroEL protein. The increased time of interaction between the mutant chaperonin and non-native proteins may explain the slower growth characteristics of CG714 compared to the parental strain B178.
drolsysis of ATP (35), together with an interaction (in E. coli)
with proteins DnaJ and GrpE (36) seem to drive the process
of polypeptide interaction. It was further concluded that BiP preferen-
tially binds protein domains exposing hydrophobic but flexible
side chains (37). Although similar experiments have not
been reported for the GroEL-related chaperonin class, there
is some preliminary evidence that hydrophobic sites may be
involved in the formation of the binary complexes between
non-native proteins and chaperonins (24). Nevertheless, polypep-
dides associated with GroEL appear to maintain a greater
amount of secondary structure relative to those bound to
DnaK (25, 28, 39). Hence, there is no guarantee that these
molecular chaperones function in a similar fashion.

In this paper, we have attempted to shed some light on the
mechanism of action of bacterial chaperonins by comparing
and contrasting the behavior of wild type GroEL and a single
amino acid substitution mutant, GroEL140. Because of the peculiar "double doughnut" organization of the GroEL-protein
(6), it is likely that even minor changes in the protomers
induce more pronounced changes on the structure and function
of the assembled molecule.

We first tested GroEL140 for its ability to hydrolyze ATP
in a magnesium-potassium-dependent manner since a weak
ATPase activity requiring these ions appears to be a typical characteristic of chaperonins. Fig. 1 shows that GroEL
GroEL140 was fully dependent upon added potassium to
achieve ATP hydrolysis. However, the ATPase activity of the
mutant was reproducibly 1.5-fold lower compared to that of
the wild type. Although we cannot rule out the possibility
that this small decrease in activity was related to the presence
of inactive enzyme in the purified GroEL140 stock solution, we
believe that this change was a direct result of the mutation
since significant structural modifications were observed in
GroEL140 by protease accessibility experiments (Fig. 2, see
below). However, the GroEL140 mutation is probably not
located in a region essential for ATP hydrolysis since the
mutant chaperonin retained ATPase activity.

Non-native proteins forming binary complexes with GroEL
can usually be discharged in a biologically active conformation
by addition of ATP and GroES (16, 20, 21, 23–27, 30). How-
ever, in some instances, non-hydrolyzable ATP analogs have
been shown to achieve the same result albeit with a lower
efficiency (24, 26, 27). ADP or the adenosine nucleotides AMP-
PNP and ATPγS were able to inhibit the ATPase activity of both
GroEL and GroEL140, suggesting that these nucleotides compete with ATP for the ATP-binding site. Surprisingly,
even though a 100-fold molar excess of nucleotides over ATP
was used in these experiments, full inhibition was only ob-
erved with ATPγS. Hence, it is likely that the latter nucleo-
tide possesses a higher affinity for GroEL. This result is
consistent with the fact that ATPγS is more efficient than
AMP-PNP at discharging GroEL-bound dihydrofolate reduc-
tase (26) as well as other model proteins. It should also be
noted that ADP inhibited the ATPase activity of both GroEL
and GroEL140 better than AMP-PNP. This implies that ADP
retains a reasonably high affinity for the ATP-binding site
and that an additional event (possibly mediated by GroES)
may be necessary to release ADP and allow ATP binding to
take place.

Several models have been proposed to describe the mode
of action of chaperonins (9, 40). They usually include a step
where the conformation of GroEL changes in order to drive
the release of the bound protein. An attractive signal for the
induction of such structural modifications is the binding and/
or hydrolysis of ATP. In fact, evidence for ATP-mediated
conformational changes in DnaK (35) and BiP (41) has been
presented. Sensitivity to proteolytic degradation can provide
information of the conformational changes undergone by a
protein if protease cleavage sites become more accessible or
are buried during the process. While purified GroEL was
essentially resistant to proteolytic degradation by trypsin
under our experimental conditions, the half-life of GroEL140
was significantly shorter, suggesting that the two proteins
adopted different conformations. Since neither the migration
pattern on non-denaturing gels or size exclusion columns, nor
the ATPase activity, nor the ability to bind unfolded polypep-
dides were significantly affected, we concluded that GroEL140
could assemble in a conformation that was similar to that of
the wild type but was probably less tightly packed as a result
of the mutation. The presence of ATP, ATP analogs, or ADP
made both proteins much more susceptible to trypsin hydro-
dysis. Therefore, the mere binding of nucleotides to GroEL
induces a topological change in the molecule, although we
cannot rule out that a subsequent conformational change is
involved. This observation is consistent with the results of
Kassenbrock and Kelly (41) who determined that nucleotide
binding but not hydrolysis alter the conformation of BiP. In contrast, DnaK conformational changes seem to be dependent upon ATP hydrolysis (35). Interestingly, the binding of GroES did not affect the trypsin digestion pattern of GroEL suggesting that (i) either the binding of the cochaperonin did not impart a significant conformational change in the GroEL-ATP(ADP) complex or (ii) that the residues affected by the binding were not recog-
nized or accessible to trypsin.

The poor efficiency of Rbu-Pz-carboxylase refolding by
GroEL140 was shown to be related to a suboptimal interaction
between the mutant chaperonin and GroES. Obviously, the
mutation affected the affinity of GroES for GroEL140 since
a stable complex between these proteins could not be isolated
by size exclusion chromatography. However, the interaction
between the two chaperonins was not completely abolished
since (i) the presence of GroES allowed the formation of a 1:2
Rbu-Pz-carboxylase dimer as judged by gel filtration chro-
matography, and (ii) increasing the contact time between
GroES and GroEL140-Rbu-Pz-carboxylase or the molar ex-
cess of GroES similarly enhanced the recovery of biologically
active Rbu-Pz-carboxylase. In agreement with the in vitro results, in vivo pulse-chase experiments showed that overex-
pressed Rbu-Pz-carboxylase remained associated with
GroEL about twice as long compared with GroEL. The mag-
nitude of the effect is comparable to the 6–7-fold lower yield
of refolded Rbu-Pz-carboxylase obtained by in vitro experi-
ments involving wild type and mutant chaperonins. Never-
thest, release of labeled Rbu-Pz-carboxylase bound to either
GroEL or GroEL140 is completed within a few minutes in
 vivo, while the process is slower in vitro. This raises the
possibility that cellular factors other than GroES could be
necessary to achieve optimal turnover of polypeptides asso-
ciated with chaperonins.

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REFERENCES
1. Ellis, R. J. (1990) Semin. Cell Biol. 1, 1–9
2. Ellis, R. J., and van der Vies, S. M. (1991) Annu. Rev. Biochem. 60, 321–347
3. Gatenby, A. A., Viitanen, P. V. and Lorimer, G. H. (1990) Trends Biotechnol. 8, 354-358
4. Rothman, J. E. (1989) Cell 59, 591-601
5. Morimoto, R., Tissieres, A., and Georgopoulos, C. (1990) in Stress Proteins in Biology and Medicine (Morimoto, R., Tissieres, A., and Georgopoulos, C., eds) pp 1-36, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
6. Hendrix, R. W. (1979) J. Mol. Biol. 129, 375-392
7. Viitanen, P. V., Lorimer, G. H., Seetharam, R., Gupta, R. S., Oppenheim, J., Thomas, J. O., and Cowan, J. N. (1992) J. Biol. Chem. 267, 695-698
8. Fayet, O., Ziegelhoffer, T., and Georgopoulos, C. (1989) J. Bacteriol. 171, 1379-1385
9. Georgopoulos, C., and Ang, D. (1990) Semin. Cell Biol. 1, 19-25
10. Fayet, O., Louarn, J. M., and Georgopoulos, C. (1986) Mol. Gen. Genet. 202, 435-445
11. Jeniexins, A. J., March, J. B., Oliver, I. R., and Masters, M. (1986) Mol. Gen. Genet. 202, 446-454
12. Miki, T., Orita, T., Furuno, M., and Horuchi, T. (1988) J. Mol. Biol. 201, 327-338
13. Phillips, G. J., and Silhavy, T. J. (1990) Nature 344, 882-884
14. Kusukawa, N., Yura, T., Ueguchi, C., Akiyama, Y., and Ito, K. (1989) EMBO J. 8, 3517-3521
15. Van Dyk, T. K., Gatenby, A. A., and LaRossa, R. A. (1989) Nature 342, 451-453
16. Viitanen, P. V., Lubben, T. H., Reed, J., Goloubinoff, P., O'Keefe, D. P., and Lorimer, G. H. (1996) Biochemistry 29, 5665-5671
17. Liessin, N. M., Vensasimov, S. Y., and Girahovich, A. S. (1990) Nature 348, 339-342
18. Tilly, K., and Georgopoulos, C. (1982) J. Bacteriol. 149, 1082-1088
19. Zweig, M., and Cummings, D. J. (1973) J. Mol. Biol. 80, 505-518
20. Goloubinoff, P., Gatenby, A. A., and Lorimer, G. H. (1989) Nature 337, 44-47
21. Goloubinoff, P., Christeller, J. T., Gatenby, A. A., and Lorimer, G. H. (1989) Nature 342, 884-889
22. Chandrasekhar, G. N., Tilly, K., Woolford, C., Hendrix, R., and Georgopoulos, C. (1986) J. Biol. Chem. 261, 12414-12419
23. Buchner, J., Schmidt, M., Fuchs, M., Jaenicke, R., Rudolph, R., Schmid, F. X., and Kiefhaber, T. (1991) Biochemistry 30, 1586-1591
24. Mendoza, J. A., Rogers, E., Lorimer, G. H., and Horowitz, P. M. (1991) J. Biol. Chem. 266, 13044-13049
25. Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A. L., and Ulrich Hartl, F. (1991) Nature 352, 36-42
26. Viitanen, P. V., Donaldson, G. K., Lorimer, G. H., Lubben, T. H., and Gatenby, A. A. (1991) Biochemistry 30, 9716-9723
27. Badco, I. G., Smith, C. J., Wood, S., Halsall, D. J., Holbrook, J. J., Lund, P., and Clarke, A. R. (1991) Biochemistry 30, 9195-9200
28. Tilly, K., Murialdo, H., and Georgopoulos, C. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 1629-1633
29. Somerville, C. R., and Somerville, S. C. (1984) Mol. Gen. Genet. 193, 214-219
30. Viitanen, P. V., Gatenby, A. A., and Lorimer, G. H. (1992) Protein Science, in press
31. Gatenby, A. A., Castleton, J. A. and Saul, M. W. (1981) Nature 291, 117-121
32. Neidhardt, F. C., Phillips, T. A., van Bogelen, R. A., Smith, M. W., Georgalis, Y., and Subramanian, A. R. (1981) J. Bacteriol. 145, 513-520
33. Tahita, F. R., and McFadden, B. A. (1974) J. Biol. Chem. 249, 3459-3464
34. Lorimer, G. H., Badger, M. R., and Andrews, T. J. (1977) Anal. Biochem. 78, 66-75
35. Liberek, K., Skowysz, D., Zylicz, M., Johnson, C., and Georgopoulos, C. (1991) J. Biol. Chem. 266, 14491-14496
36. Liberek, K., Marezalek, J., Ang, D., and Georgopoulos, C. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2874-2878
37. Flynn, G. C., Pohl, J., F scoo, M. T., and Rothman, J. E. (1991) Nature 353, 726-730
38. Landry, S. J., and Giers, L. M. (1991) Biochemistry 30, 7559-7562
39. van der Vies, S. M., Viitanen, P. V., Gatenby, A. A., Lorimer, G. H., and Jaenicke, R. (1992) Biochemistry, in press
40. Creighton, T. F. (1991) Nature 352, 17-18
41. Kassenbrock, C. K., and Kelly, R. B. (1989) EMBO J. 8, 1461-1467