Functional Characterization of an Archaeal GroEL/GroES Chaperonin System

SIGNIFICANCE OF SUBSTRATE ENCAPSULATION*

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In all three kingdoms of life chaperonins assist the folding of a range of newly synthesized proteins. As shown recently, Archaea of the genus Methanosarcina contain both group I (GroEL/GroES) and group II (thermosome) chaperonins in the cytosol. Here we report on a detailed functional analysis of the archaeal GroEL/GroES system of Methanosarcina mazei (Mm) in comparison to its bacterial counterpart from Escherichia coli (Ec). We find that the groESgroEL operon of M. mazei is unable to functionally replace groESgroEL in E. coli. However, MMGroES protein can largely complement a mutant EcGroES protein in vivo. The ATPase rate of MMGroEL is very low and the dissociation of MMGroES from MMGroEL is 15 times slower than for the EcGroEL/GroES system. This slow ATPase cycle results in a prolonged enclosure time for model substrate proteins, such as rhodanese, in the MMGroEL/GroES folding cage before their release into the medium. Interestingly, optimal functionality of MMGroEL/GroES and its ability to encapsulate larger proteins, such as malate dehydrogenase, requires the presence of ammonium sulfate in vitro. In the absence of ammonium sulfate, malate dehydrogenase fails to be encapsulated by GroES and rather cycles on and off the GroEL trans ring in a non-productive reaction. These results indicate that the archaeal GroEL/GroES system has preserved the basic encapsulation mechanism of bacterial GroEL and suggest that it has adjusted the length of its reaction cycle to the slower growth rates of Archaea. Additionally, the release of only the folded protein from the GroEL/GroES cage may prevent adverse interactions of the GroEL substrates with the thermosome, which is not normally located within the same compartment.

A subset of newly synthesized proteins in the cytosol, as well as in mitochondria and chloroplasts, depend on chaperonins, a family of structurally related molecular chaperones, for folding assistance (1–9). In contrast to other types of molecular chaperones that act more generally in de novo folding, such as the Hsp70s, the chaperonins form large cylindrical double-ring structures in which a single molecule of unfolded protein is transiently enclosed and allowed to fold unimpeded by aggregation. The chaperonins have been divided into two distinct classes, group I and group II, with members of both groups exhibiting only limited sequence homology but an overall similar architecture of the oligomeric ring complexes (8–13).

Group I chaperonins, also known as Hsp60s or Cpn60s, are generally found in the bacterial cytosol (e.g. GroEL in Escherichia coli) as well as in mitochondria (mtHsp60) and chloroplasts (Rubisco subunit binding protein), and typically form homo-oligomeric complexes of two stacked heptameric rings. They cooperate functionally with cofactors of the Hsp10 (GroES) family, single heptameric rings of ~10-kDa subunits that bind to the ends of the Hsp60 cylinder. GroEL of E. coli and its cofactor GroES have been extensively analyzed in terms of their structure and function. GroEL is composed of 14 identical 57-kDa subunits. Each subunit consists of three domains: the equatorial domain contains the ATP binding site and mediates most intersubunit contacts within and between rings. It is connected via an intermediate hinge-like domain to the apical domain. The apical domains expose a number of hydrophobic residues toward the ring cavity for the binding of unfolded protein substrate and provide binding regions for flexible sequence loops on the subunits of GroES.

The ATP-dependent interactions of GroEL with protein substrate and GroES have been studied extensively (reviewed in Refs. 2–4, 6, and 7). Briefly, binding of 7 ATP to GroEL results in the displacement of GroEL-bound substrate protein into an enclosed folding cage, the so-called cis complex. Proteins up to ~60 kDa can be become enclosed and fold in the confined environment of the cage in the time it takes the 7 ATP to be hydrolyzed to ADP (~10–15 s at 25 °C). Upon completion of this first round of ATP hydrolysis, binding of 7 ATP to the opposite ring of GroEL (the trans ring) transmits an allosteric signal to the cis ring, causing the dissociation of the 7 ADP and GroES. At this point, folded protein leaves the cage, whereas incompletely folded intermediates are rapidly recaptured by the same or another GroEL complex for a subsequent folding

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Recently, it has been shown that GroEL can assist in the folding of certain proteins too large to be enclosed by GroES using a mechanism that involves cycling on and off the trans ring (14, 15).

In contrast to the group I chaperonins, the group II chaperonins of the archaeal cytosol (known as thermosome) are composed of 3- to 3 types of different subunits and form double-ring structures with 8- or 9-fold symmetry (16). Significantly, greater structural complexity is exhibited by the group II chaperonin of the eukaryotic cytosol (known as CCT for chaperonin containing TCP-1 or as TRiC for TCP-1 ring complex), which consists of eight orthologous subunits per ring. The group II chaperonins are generally independent of a Hsp10 (GroES)-like cofactor but cooperate with the molecular chaperone prefoldin (also called GimC) in Archaea and eukaryotes (17–21). Their mechanism of action involves protein encapsulation by helical extensions of the apical chaperonin domains that provide a “built in” GroES function (22).

It has generally been assumed that groups I and II chaperonins do not co-exist in the same cellular compartment. However, the recent genome sequencing of three mesophilic archaeae, Methanosarcina barkeri (M. barkeri; ~2.8 Mbp, US DOE Joint Genome Institute), Methanosarcina acetivorans (M. acetivorans, ~5.8 Mbp) (23), and Methanosarcina mazei Gs1 (M. mazei, ~4.1 Mbp) (24) revealed the presence of both group I and group II chaperonin genes that are simultaneously expressed to similar levels in the cytosol (25). The M. mazei (Mm) and E. coli (Ec) GroEL/GroES systems exhibit 54% sequence identity and 72% similarity.

So far, almost all our knowledge of the group I chaperonin mechanism has come from studies of the bacterial EcGroEL/ GroES system. A functional analysis of the archaeal MmGroEL/GroES system in comparison to its bacterial counterpart now offers the opportunity to define general features of group I chaperonin function that have been conserved in evolution. Moreover, it may provide insight into how the archaeal MmGroEL/GroES system functionally adapted because of co-evolution with the group II chaperonin. Here we show that MmGroEL/GroES is unable to functionally replace EcGroEL/ GroES in E. coli. However, the MmGroES protein can largely complement a mutant EcGroES protein in vitro. The ATPase rate of MmGroEL is very low and the dissociation of MmGroES from MmGroEL is 15 times slower than for the EcGroEL/ GroES system. Indeed, model substrates such as rhodanese accumulate in a folded state inside the MmGroEL/GroES folding cage before they are released into the medium. Optimal functionality of MmGroEL/GroES and substrate encapsulation requires the presence of ammonium sulfate. Interestingly, in the absence of ammonium sulfate, stringently GroEL-dependant proteins such as malate dehydrogenase fail to be encapsulated by GroES and rather cycle on and off the GroEL trans ring in a non-productive reaction. These results indicate that the archaeal GroEL/GroES system has preserved the basic encapsulation mechanism of bacterial GroEL and suggest that it has adjusted the length of its reaction cycle to the slower growth rates of Archaea. Additionally, the release of only folded protein from the GroEL/GroES cage would avoid non-productive interactions of the GroEL substrates with the thermosome, which is not normally located within the same compartment.

**EXPERIMENTAL PROCEDURES**

**Proteins**

E. coli GroEL and GroES and M. mazei GroEL and GroES were purified as described (25). EcGroES and MmGroES were cloned into pET-22b (Novagen) resulting in C-terminal His-tagged constructs, allowing immobilization on the NTA (nitrilotriacetate) biosensor chip. The His-tagged GroES proteins were overexpressed by induction in BL21 (DE3) with 1 mM isopropyl-β-D-galactopyranoside. Supernatants were applied on Ni²⁺-NTA (Qiagen) and the GroES fractions were further purified by chromatography on Superdex 200 (Amersham Biosciences) in a buffer composed of 20 mM MOPS, pH 7.4, 100 mM KCl, 10 mM MgCl₂. Bovine mitochondrial rhodanese and mitochondrial malate dehydrogenase (MDH) from porcine heart were purchased from Sigma. Protein concentrations were determined using calculated molar extinction coefficients at 280 nm of 9080 M⁻¹cm⁻¹ for EcGroEL, 1,280 M⁻¹cm⁻¹ for EcGroES, 10,360 M⁻¹cm⁻¹ for MmGroEL, 5120 M⁻¹cm⁻¹ for MmGroES, 59,840 M⁻¹cm⁻¹ for rhodanese; 7,680 M⁻¹cm⁻¹ for MDH. The antibodies against rhodanese and MDH were prepared by immunizing rabbits with a 1:1 emulsion of the purchased proteins with Freund’s adjuvant (Sigma).

**Bacterial and Bacteriophage Genetic Manipulations**

The parental E. coli strain used in this study is B178, a W3110 galE sup derivative. B178 and MC4100 araD714 have been previously described (26–28). The mutants groEL44 and groES619 and all the bacteriophages used in this study have also been described (26, 27, 29, 30). The EcgroES and EcgroESgroEL genes were cloned under the arabinose-inducible promoter of pBAD22 (31), using standard molecular cloning techniques (a kind gift of Dr. France Keppel). The MmgroES-groEL operon was amplified from genomic libraries of M. mazei Gs1 (24) by PCR using primers described in Ref. 25 and inserted into pET22b using NdeI and BamHI restriction sites. The genes MmgroES, MmgroEL, and EcgroESgroEL together with the ribosomal binding site of the corresponding pET22b (Novagen) plasmids (25) were excited with XbaI and HindIII and inserted into the arabinose-inducible promoter of pBAD18 (31).

Classical bacteriophage P1-mediated transduction was used to determine whether the MmGroESgroEL operon can replace that of E. coli. A P1 lysate was grown on E. coli strain AR189 (27) whose chromosomally encoded groESgroEL operon has been deleted and substituted with a chloramphenicol resistance (Cam) encoding cassette. This Cam'- encoding cassette is ~50–60% linked to a nearby Tn10 transposon, encoding resistance to tetracycline (Tet'), i.e. when one selects first for the Tet' marker in the recipient strain, then ~50% of such transductants should simultaneously become Cam', provided the essential EcgroESgroEL operon (32) is carried on a plasmid. Accordingly, the bacteriophage P1 lysate grown on strain AR189 was used to infect wild-type E. coli. B178 strains carrying various plasmid constructs (Tables I and II). Tet' transductants were selected on LB-agar plates supplemented with 12.5 μg/ml tetracycline and 5 mM sodium citrate (to prevent re-infection and killing by the P1 bacteriophage). Following incubation for 2 days at 30 °C, Tet' colonies were tested for inheritance of the Cam' marker by cross-streaking the Tet' transductants on LB-agar plates supplemented with 5 mM sodium citrate and either 12.5 μg/ml tetracycline or 10 μg/ml chloramphenicol, followed by incubation overnight at 30 °C.

Genetic complementation experiments were carried out as follows: isogenic E. coli wild-type MC4100 or groES619 or groEL44 bacterial strains were first transformed with a series of pBAD constructs created by standard PCR methods carrying various combinations of the E. coli (Ec) and M. mazei (Mm) genes for selecting for plasmid-encoded ampicillin resistance (Amp') at 30 °C. Bacterial transformants were grown at 30 °C overnight in LB broth supplemented with 100 μg/ml ampicillin without arabinose. For complementation of the bacterial temperature-sensitive phenotype at 43 °C, bacterial cultures were serially diluted in cold LB broth and 0.3-ml aliquots of overnight cultures were spread over LB-agar plates supplemented with 0.1% arabinose and 100 μg/ml ampicillin and incubated overnight at 30 and 43 °C for 16 h. For complementation of bacteriophage λ, T4 or T5 growth, 0.3-ml aliquots of overnight cultures in LB broth supplemented with 100 μg/ml ampicillin were added to 3 ml of LB soft agar (0.66% agar) and evenly distributed over LB-agar plates containing 0.1% arabinose. When the soft agar solidified, 10-fold dilutions of various bacteriophage lysates were spotted on the bacterial lawn and, following drying of the plates, incubated at 37 °C for 16 h.

**ATPase Assay**

GroEL (1 μm oligomer) was added to Buffer A (20 mM MOPS, pH 7.5, 100 mM KCl, 5 mM MgCl₂) or Buffer A, 0.5 mM ammonium sulfate (AS) and incubated for 5 min at 37 °C. Where indicated, GroES was present at a 2-fold molar excess over GroEL. The reaction was initiated at 37 °C by the addition of 2 mM ATP. The kinetics of the ATPase activities of EcGroEL and MmGroEL were followed for 0-5 and 0–25 min, taking time points every 1 and 5 min, respectively. ATPase activity at the various time points was stopped by the addition of ~15 mM CDTA. Quantification of liberated inorganic phosphate was performed by the
malachite green assay (33) after incubation for 30 min at 25°C, and absorption was measured at 640 nm. A calibration curve of inorganic phosphate (0–20 nmoi) was measured in parallel. No spontaneous ATP hydrolysis was observed under the conditions used.

Refolding Assays

Rhodanese Refolding—Rhodanese was denatured by incubation for 30 min at 37°C in denaturing buffer (6 M guanidinium HCl, 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM MgCl₂, and 5 mM dithiothreitol) and diluted 100-fold into Buffer A or Buffer A, 0.5 mM AS at 37°C in the absence or presence of various concentrations of the chaperonins (as specified in the figure legends). At the times indicated, chaperonin action was stopped with CDTA (50 mM) and rhodanese activity measured at 460 nm at 25°C, as previously described (34, 35).

Malate Dehydrogenase Refolding—MDH was denatured for 30 min in Buffer A containing 3 mM guanidinium HCl, 5 mM dithiothreitol and diluted 100-fold into Buffer A or Buffer A, 0.5 mM AS at 37°C in the absence or presence of various concentrations of the chaperonins (as specified in the figure legends). At the times indicated, aliquots were withdrawn and chaperonin-assisted refolding stopped by the addition of 50 mM CDTA. Enzyme activities were measured essentially as described (36, 37) at 25°C in assay mixture (Buffer A, 1 mg/ml bovine serum albumin (Sigma), 0.22 mM β-NADH (Sigma), 0.55 mM oxaloacetate (Sigma), 1 mM CDTA), after 60 min incubation at 20°C. The time dependent oxidation of β-NADH was monitored for ~1 min at 340 nm.

Size Exclusion Chromatography

At 5 and 45 min into the chaperonin-assisted refolding assays of rhodanese or MDH, an aliquot of 50 μl was withdrawn from the reaction and further ATP hydrolysis was inhibited by the addition of 25 μM glucose and 0.3 units/μl hexokinase (Roche Diagnostics). The sample was then applied onto a Superose 6 3.2/30 (Amersham Biosciences) size exclusion column equilibrated in Buffer A. Fractions were analyzed for enzyme activity and/or resolved by 16% SDS-PAGE followed by Coomassie Blue staining for GroEL, whereas the other half was fluorodated for GroEL/GroES antibodies. Protease Protection

MDH was denatured as described above and diluted 100-fold into Buffer A or Buffer A, 0.5 mM AS in the presence of an equimolar concentration of GroEL chaperonin at 37°C. Protein aggregates were removed by centrifugation for 10 min at 10,000 × g. The supernatant was divided into two equal portions, one portion receiving 4 mM AMP-PNP (or 2 mM ADP) and the other 4 mM AMP-PNP (or 2 mM ADP), 1 μM GroES, followed by incubation for 5 min. Treatment with 1.5 μg/ml proteinase K (from Trichirachium album, Roche) was followed for 0–15 min at 25°C and the proteinase K action stopped with 1 mM phenylmethylsulfonyl fluoride. Half of each reaction was resolved by 8% SDS-PAGE followed by Coomassie Blue staining for GroEL, whereas the other half was resolved by 16% SDS-PAGE and transferred to nitrocellulose and immunoblotted with anti-rhodanese or anti-MDH antibodies.

Surface Plasmon Resonance

EcGroES and MmGroES, modified with a C-terminal His₁₅ tag, were immobilized (~30 response units) on a chelating NTA biosensor chip (BIAcore Inc. (38)) using a BIAcore 2000 instrument at 37°C. Approximately 20–40% of the immobilized GroES was competent in GroEL binding. Association and dissociation of the GroEL chaperonins in Buffer A, 2 mM ATP was performed for 480 s at a flow rate of 20 μl/min, with an association phase for 8 s followed by dissociation for 15–20 min. Kinetic analyses with 25 to 500 nM of the GroEL chaperonins were performed essentially as described in Ref. 39 using BIAevaluation software 3.2.

Miscellaneous

The following procedures were carried out according to published methods: SDS-PAGE (40), electrotransfer to nitrocellulose (41), immunoblotting with primary polyclonal antibody was carried out in TBS-T (25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.05% Tween 20) followed by incubation with secondary antibody (horseradish peroxidase-conjugated, anti-rabbit, Sigma) and developed using ECL reagents (Amer sham Biosciences) (42).

RESULTS

Partial Exchangeability of Components between the Archaeal and Bacterial GroEL/GroES Systems in Vitro—The monomeric model substrate rhodanese (~35 kDa) has been shown to require the EcGroEL/GroES system and ATP for successful refolding from denaturant at concentrations where the unfolded protein tends to aggregate in the absence of chaperones. In a recent study we showed that the archaeal group I chaperonin of M. mazei, MmGroES, is as efficient as EcGroEL in preventing the aggregation of denatured rhodanese (25). This effect was optimal at a nearly equimolar ratio of MmGroEL to rhodanese. Refolding of rhodanese by MmGroEL/MmGroES, as followed by the regain of enzymatic activity, occurred with somewhat slower kinetics but the same final yield as with EcGroEL/EcGroES and was dependent on the complete chaperonin system, that is, MmGroEL, MmGroES, and hydrolyzable ATP (Ref. 25 and Fig. 1). MmGroES was found to functionally cooperate with EcGroEL, resulting in rhodanese refolding with similar overall kinetics as those observed with MmGroEL/MmGroES but with a ~25% lower yield (Fig. 1). Surprisingly, the combination of MmGroEL with EcGroES proved to be completely inactive in rhodanese refolding (Fig. 1).

As will be shown below, this is because of the inability of EcGroEL in preventing the aggregation of denatured rhodanese (25). We have previously shown that the EcGroES(EcGroEL) operon cannot replace E. coli groES(EcGroEL) operon in vivo. The ability to cotransduce the chromosomally encoded Tet<sup>α</sup> and Cam<sup>α</sup> alleles indicates that the plasmid-encoded genes can replace the groES(EcGroEL) operon of E. coli. For details, see “Experimental Procedures” and “Results.”

| E. coli recipient | Tet<sup>α</sup> | Tet<sup>α</sup> Cam<sup>α</sup> | Cotransduction |
|-------------------|--------------|------------------|----------------|
| B178 (pBAD22Ecgro) | 61           | 34               | 56             |
| E. coli (EcgroEL<sup>α</sup>) | 88<sup>a</sup> | 49<sup>a</sup> | 56<sup>a</sup> |
| B178 (pBAD18Mmgro) | 72           | 0                | 0              |
| E. coli (MmGroES<sup>α</sup>) | 51<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> |

<sup>a</sup> Anaerobic conditions (37°C, 48 h, 5% CO₃N₂)
In contrast, temperature of 43 °C was tested under the same conditions but at 43 °C (see “Experimental Procedures” for details). *++,* wild type size colonies or plaques, with an efficiency of plating of ~1.0; ††, good growth, but colonies or plaques smaller than wild type, but still with an efficiency of plating of ~1.0; †, some growth, but colonies or plaques are very small, with an efficiency of plating of ~0.1; 0, no detectable colony or plaque formation, with an efficiency of plating less than 0.001.

| Bacterial growth at 43 °C with 0.1% arabinose | λ | T5 | T4 |
|-----------------------------------------------|---|----|----|
| MC4100 (pBAD18)                               | ++|++ |++ |
| MC4100 (pBAD18MmgroESgroEL)                   | ++|++ |++ |
| MC4100 (pBAD18MmgroES)                        | ++|++ |++ |
| MC4100 (pBAD22MmgroES)                        | ++|++ |++ |
| MC4100 groES619 (pBAD18)                      | ++|++ |++ |
| MC4100 groES619 (pBAD18MmgroESgroEL)          | ++|++ |++ |
| MC4100 groES619 (pBAD18MmgroES)               | ++|++ |++ |
| MC4100 groES619 (pBAD18MmgroESgroEL)          | ++|++ |++ |
| MC4100 groEL44 (pBAD18MmgroESgroEL)           | ++|++ |++ |
| MC4100 groEL44 (pBAD18MmgroES)                | ++|++ |++ |
| MC4100 groEL44 (pBAD18MmgroES)                | ++|++ |++ |
| MC4100 groEL44 (pBAD22MmgroES)                | ++|++ |++ |
| MC4100 groEL44 (pBAD18MmgroES)                | ++|++ |++ |
| MC4100 groEL44 (pBAD18MmgroES)                | ++|++ |++ |
| MC4100 groEL44 (pBAD18MmgroES)                | ++|++ |++ |
| MC4100 groEL44 (pBAD22MmgroES)                | ++|++ |++ |

Tet<sup>+</sup> transductants simultaneously inherited the nearby Cam<sup>R</sup>-encoding cassette (Table I). In contrast, when the *E. coli* recipient strain carried a plasmid encoding the MmgroES-groEL operon, none (0/72) of the Tet<sup>+</sup>-encoding transductants simultaneously inherited the nearby Cam<sup>R</sup>-encoding allele. Because *M. mazei* is a strictly anaerobically growing organism, this same type of P1 transduction experiment was repeated under anaerobic growth conditions. Table I shows that, even under these conditions, the MmgroESgroEL operon failed to replace its EcgroESgroEL counterpart.

The MmgroES Gene Can Functionally Replace Its *E. coli*-encoded Homologue—Following our failure to substitute the entire groESgroEL operon of *E. coli* by that of *M. mazei*, we asked whether the individual MmgroES or MmgroEL genes can functionally complement their counterparts in *E. coli*. This was done by transforming isogenic *E. coli* strains, either wild-type or carrying the groES619 or groEL44 mutations, with plasmids encoding various constructs of wild-type genes from either *E. coli* or *M. mazei* (Table II). *E. coli* carrying either the groES619 or groEL44 mutant alleles cannot propagate the λ and T5 bacteriophages or form colonies at 43 °C (26, 44). In addition, groEL44 mutant bacteria cannot propagate bacteriophage T4 at 37 °C (Table II). The following observations were made: (a) the MmgroES wild-type gene complemented the groES619 defect in bacterial growth at the nonpermissive temperature of 43 °C and the growth of bacteriophage T5 at 37 °C. In contrast, MmgroES did not efficiently substitute for EcgroES in bacteriophage λ growth at 37 °C. These results indicate that the MmgroES protein can indeed carry out most of the essential in vivo functions of EcGroES. Surprisingly, expression of the MmgroES gene in trans could also largely correct many of the defects exhibited by the groEL44 mutation, namely bacterial temperature sensitivity at 43 °C and resistance to bacteriophages λ and T5 at 37 °C. As expected, MmgroES did not correct bacteriophage T4 growth at 37 °C because T4 needs a bacteriophage-encoded GroES-like chaperone (Gp31) for the proper processing of its Gp23 capsid protein (43–45). (b) In contrast to the MmgroES results, the expression of the MmgroEL protein in trans did not significantly correct any of the defects exhibited by groEL44 mutant bacteria in terms of bacteriophage or bacterial growth (Table II). In sharp contrast to MmgroES, expression of MmgroEL from a multicopy plasmid, either alone or in combination with MmgroES, interfered with *E. coli* growth at 43 °C (Table II) and partially with bacteriophage T4 growth at 37 °C.
Fig. 3. Retarded release of active rhodanese from the MmGroEL/GroES chaperonin cavity during chaperonin assisted refolding. Analysis of samples at the 5-min time point into the chaperonin-assisted refolding from Fig. 1 by size exclusion chromatography and assaying the rhodanese activity of the corresponding fractions. The trace of absorbance at 280 nm indicates the position of the chaperonin complexes in the size exclusion chromatography. The gray bars reflect the amount of native rhodanese in the respective column fractions as measured by the rhodanese activity assay.

Fig. 4. The M. mazei chaperonin system binds MDH but fails to mediate its refolding under standard buffer conditions. A, analysis of MDH refolding. Chemically denatured MDH (50 μM) was diluted 100-fold into either Buffer A (▼) or Buffer A containing either 1 μM EcGroEL, 2 μM EcGroES (○) or 1 μM MmGroEL, 2 μM MmGroES (□) at 37 °C. Folding reactions were initiated by the addition of 5 mM ATP. The MDH activity is shown as a percentage of the activity obtained with 0.5 μM native MDH. At 5 and 45 min into the assisted refolding, aliquots were withdrawn and analyzed by size exclusion chromatography as described in the legend to Fig. 3. B, analysis of the fractions from the size exclusion chromatography at the 5- and 45-min time points of chaperonin-assisted refolding. Fractions from the size exclusion chromatography were resolved by 16% SDS-PAGE and immunoblotted with anti-MDH. The position of the chaperonin complexes was determined by Coomassie Blue staining of the same fractions following 16% SDS-PAGE analysis (data not shown) and is indicated by the bracket from fractions 7–10.

In summary, these in vivo studies demonstrate that the MmGroES protein can complement in vivo for most of EcGroES functions. Furthermore, MmGroES must exhibit a higher overall affinity for EcGroEL because it efficiently suppressed the defects of the EcGroEL44 mutant protein, previously shown to exhibit diminished ability to bind to its cochaperone (44, 45).

The Archaeal and Bacterial GroEL/GroES Systems Cycle with Different Kinetics—In vitro experiments were performed to explore the possible basis for the functional differences between the archaean and bacterial chaperonin systems. One parameter that influences the production of refolded protein by chaperonin is the rate of GroES cycling on and off GroEL. Surface plasmon resonance (SPR) experiments were performed by immobilizing C-terminal His-tagged EcGroES or MmGroES on a NTA biosensor chip. Dissociation rates were measured with the two forms of GroEL in the presence of ATP at 37 °C. Under the experimental conditions used, EcGroEL dissociated from EcGroES with a rate constant of $4.1 \times 10^{-3}$ s$^{-1}$ (Fig. 2A), corresponding to a half-time for dissociation of $35$ s. Interestingly, MmGroEL was found to dissociate from immobilized MmGroES $\sim 15$ times more slowly, with a rate constant of $2.6 \times 10^{-3}$ s$^{-1}$ (Fig. 2B). Because the folded subunits of oligomeric substrate proteins can associate and become biologically active only after leaving the GroEL/GroES cage, the M. mazei chaperonin system would be about $15$ times less efficient in supplying growing cells with active enzyme. This difference may provide an explanation for the failure of the M. mazei system to replace the corresponding E. coli system in vivo (Table I).

The results of the SPR analysis also readily explain the failure of MmGroEL to cooperate with EcGroES in rhodanese refolding. As shown in Fig. 2A, MmGroEL does not bind to EcGroES, whereas EcGroEL binds to MmGroES and is released in the presence of ATP with the same slow kinetics as observed for MmGroES (Fig. 2B). This finding indicates that the intrinsic binding properties of MmGroES alone must contribute significantly to the slow cycling rate on and off GroEL. Moreover, the SPR measurement demonstrates that MmGroES has a higher affinity for EcGroEL than EcGroES, consistent with the observation in vivo that MmGroES complements E. coli mutants groES619 and groEL44 (Table II).
**MmGroEL Has a Much Lower ATPase Activity Than EcGroEL**—The rate of GroES cycling on and off GroEL is linked with the ATPase activity of GroEL. We therefore compared the ATPase activity of MmGroEL to that of EcGroEL. The MmGroEL oligomer has an ATPase activity (~4.5 ATP min⁻¹) ~20 times lower than its bacterial homologue (Fig. 2C), consistent with the ~15 times slower rate of GroEL/GroES cycling measured for the *M. mazei* chaperonin system by SPR (Fig. 2B). As reported previously for EcGroEL/GroES, binding of GroES inhibits the ATPase activity of GroEL by ~50% (34, 46). Interestingly, MmGroES has a much stronger effect than EcGroES on reducing the ATPase activity of EcGroEL (Fig. 2C), consistent with the observation that the off-rate of EcGroEL from MmGroES is much slower than that for EcGroES.

The *Archaeal Chaperonin Refolds Rhodanese But Releases It Very Slowly*—Both the SPR kinetic analyses and the ATPase assays indicated a slower cycling of the archaeal chaperonin system compared with its bacterial counterpart. Nevertheless, MmGroEL/GroES appears to support the folding of rhodanese to its enzymatically active state as efficiently as EcGroEL/GroES, as shown in Fig. 1.

It seemed possible that rhodanese accumulates inside the MmGroEL/GroES complex in its active form but is not readily released into the medium. We therefore investigated the release of rhodanese from the GroEL cavity during ongoing refolding with MmGroEL/GroES or EcGroEL/GroES. Refolding reactions were stopped after 5 min by the addition of glucose/hexokinase, to rapidly convert the ATP in the reaction to ADP, thereby arresting the chaperonin as a stable GroEL-GroES-ADP complex. The reactions were then analyzed by size exclusion chromatography and rhodanese enzyme activities were determined in the fractions. Considerably more rhodanese activity co-eluted with the MmGroEL/GroES complex than with EcGroEL/GroES, indicating that the MmGroEL/GroES system releases folded rhodanese more slowly than EcGroEL/GroES (Fig. 3). It has been shown previously that monomeric rhodanese acquires full enzymatic activity while being enclosed in the GroEL/GroES cage (35, 47).

**MmGroEL/GroES Fails to Produce Active, Dimeric Malate Dehydrogenase**—To further investigate the mechanism of the archaean chaperonin system, we used the dimeric protein MDH (~45 kDa subunits) as a stringently GroEL-dependent protein. This enzyme is known to require the chaperonin for successful refolding from denaturant, as shown with EcGroEL/GroES. To further investigate the mechanism of the GroEL/GroES system, we used the dimeric protein MDH (~45 kDa subunits) as a stringently GroEL-dependent protein. This enzyme is known to require the chaperonin for successful refolding from denaturant, as shown with EcGroEL/GroES. Refolding reactions were stopped after 5 and 45 min and subjected to size exclusion chromatography, followed by SDS-PAGE analysis of the fractions and immunoblotting for MDH. Even after 45 min virtually all MDH was still bound to MmGroEL/GroES, whereas free native MDH was observed with EcGroEL/GroES already after 5 min of refolding (Fig. 4B).

Ammonium Sulfate Enhances the ATPase Activity of MmGroEL and the Release of Folded Substrate—We next looked for conditions that could enhance the ATPase activity and therefore the refolding activity of the MmGroEL/GroES system. It had been reported that AS has a stimulatory effect on the ATPase of both EcGroEL (48) and the archaean thermosome from *Methanopyrus kandleri* (49). Indeed, titration experiments showed that a concentration of 0.5 M AS was optimal in stimulating the ATPase activity of MmGroEL (data not shown), more than doubling the rate compared with conditions in the absence of AS (Fig. 5A). This stimulated ATPase activity was inhibited by GroES, suggesting that AS did not cause a functional uncoupling of the GroEL ATPase. Notably, the AS-stimulated ATPase activity was still 5-fold slower than the unstimulated rate of EcGroEL.

SPR experiments revealed that in the presence of 0.5 M AS, MmGroEL dissociated from MmGroES with a rate of ~1.1 × 10⁻² s⁻¹, i.e. about 4 times faster than in its absence (Fig. 5B). This dissociation rate is ~4-fold slower than that observed for the dissociation rate of EcGroEL from EcGroES (~4.1 × 10⁻² s⁻¹) measured in the absence of AS (Fig. 5C). Approximately 2-fold higher levels of EcGroEL binding to EcGroES were observed in the presence of AS, presumably because AS increased the association rate for EcGroEL binding more strongly than the dissociation rate, which was accelerated by a factor of ~2.5 (Fig. 5C).

The presence of AS caused a slight increase in the rate of rhodanese folding for both GroEL/GroES systems, whereas the yield of refolding remained unchanged (Fig. 6A). Analysis by
sized exclusion chromatography showed that AS also accelerated the release of folded rhodanese from MmGroEL/GroES (Fig. 6B). Strikingly, in the presence of AS the archaeal chaperonin system also supported the production of active dimeric MDH at a rate similar to that observed with EcGroEL/GroES in the absence of AS (Fig. 7A). Interestingly, the rate of MDH renaturation by EcGroEL/GroES was also significantly enhanced by AS and an increase in refolding yield was consistently observed under these conditions. The basis for this effect remains to be investigated but it is likely because of an increased efficiency of GroEL/GroES function. An effect on MDH itself cannot be ruled out, however, although AS did not measurably improve the ability of MDH to refold spontaneously (Fig. 7A).

Analysis of MDH refolding reactions with MmGroEL/GroES in the presence and absence of AS demonstrated that the production of free MDH was strictly dependent on AS (Fig. 7B). Release from GroEL allows dimerization of folded monomers and subsequent acquisition of MDH activity.

Based on the result obtained with rhodanese (Fig. 3), it was possible that the inability of MmGroEL/GroES to assist MDH refolding in the absence of AS resulted from inefficient release of folded MDH from the cis cavity of the MmGroEL-GroES complex. To address this possibility, we performed protease K protection assays to investigate whether MDH was enclosed in the GroEL-GroES complex under these conditions (35, 50, 51). As expected, addition of protease K to GroEL-bound MDH in the absence of GroES resulted in the rapid digestion of the unfolded protein, independent of the presence of AS (Fig. 8, A–D, left panels). When EcGroES was added to the EcGroEL-MDH complex in the presence of the non-hydrolysable ATP analog AMP-PNP, with or without AS, a substantial amount of the bound MDH was protected against proteolysis, indicating that this protein was encapsulated in the EcGroEL/GroES cage (Fig. 8, A and B, right panels). The same result was obtained when the experiment was performed in the presence of ADP (data not shown). In contrast, addition of MmGroES to MmGroEL-MDH under the same conditions resulted in MDH protection only when AS was also added (Fig. 8, C and D, right panels). In the absence of AS, virtually all the MDH protein was digested by protease K and was therefore bound to the trans ring of the MmGroEL-GroES complex (see also Fig. 4B). MmGroES was indeed bound to MmGroEL in trans to MDH, because half of the GroEL subunits were protected against cleavage of their flexible C-terminal sequences by protease K (50), as revealed by SDS-PAGE and Coomassie Blue staining (Fig. 8E, right panel). In contrast, in the absence of GroES, the majority of GroEL was C-terminal truncated under the conditions of mild proteolysis used (Fig. 8E, left panel). These effects were independent of the presence of AS.

MDH Bound in Trans to MmGroEL/GroES Can Refold upon Addition of Ammonium Sulfate or Transfer to the Bacterial Chaperonin System—Having established that MDH is bound to the trans ring of MmGroEL/GroES in the absence of AS, we next asked whether this MDH is cycling on and off the trans ring in the presence of hydrolysable ATP. To address this possibility, we added an excess of EcGroEL and EcGroES 5 min into a refolding reaction of MDH with MmGroEL/GroES in the absence of AS. The MDH bound to the trans ring of MmGroEL:GroES was readily transferred to the bacterial chaperonin system and refolded with the same efficiency as with EcGroEL/GroES alone (Fig. 9; see also Fig. 7A). A similar result was obtained when EcGroEL/GroES was added after 15 min, albeit the final yield of refolding was ~10% lower (data not shown), apparently because prolonged trans cycling of MDH on and off MmGroEL/GroES results in a loss of refolding competence.
Thus, in the absence of AS, MDH cycles from the trans ring of MmGroEL but does not refold. Interestingly, when instead of the bacterial chaperonin, AS was added to a reaction containing MmGroEL, MmGroES, MDH, and ATP after 5 or 15 min incubation, MDH refolding was observed at a rate and yield similar to that measured when AS was present at the onset of the reaction (Fig. 9). Thus, addition of AS initiates encapsulation of MDH in the MmGroEL/GroES cage and this results in efficient refolding.

**DISCUSSION**

Thus far, three species of the archaeal genus *Methanosarcina* have been found to contain both group I (GroEL/GroES) and group II (thermosome) chaperonins in the cytosol (25). The groESgroEL operon is believed to have been acquired by these species by lateral gene transfer from an unknown bacterial source, along with ~30% of the *M. mazei* genome (24). To gain insight into how GroEL/GroES may have adapted to this unusual biological context, we have performed a detailed functional analysis of the *M. mazei* GroEL/GroES system in comparison with that of *E. coli*. Consistent with a significant diversion from its bacterial ancestor, the archaeal groESgroEL operon failed to replace the *E. coli* groESgroEL operon. As a likely explanation for this finding, our measurements showed that MmGroEL has a much slower ATPase activity and a correspondingly slower cycling rate for GroES than the bacterial system. This results in a prolonged enclosure time for the unfolded protein substrate in

**FIG. 7.** The MmGroEL/GroES system successfully refolds denatured MDH in the presence of 0.5 M ammonium sulfate. A, time course of MDH refolding at 37 °C in the absence or presence of 0.5 M AS. Chemically denatured MDH (50 µM) was diluted 100-fold into reactions containing 0.5 µM EcGroEL, 1.0 µM EcGroES or 0.5 µM MmGroEL, 1.0 µM MmGroES in the absence (○, □) or presence (●, ■) of 0.5 M AS. Folding reactions were initiated by the addition of 5 mM ATP. MDH activity is shown as a percentage of the activity obtained with 0.5 µM native MDH in Buffer A. Five min into refolding, aliquots were withdrawn and applied to a Superose 6 size exclusion column as described under “Experimental Procedures.” B, analysis of samples at the 5-min time point of MmGroEL/GroES refolding in the absence or presence of 0.5 M AS from A by size exclusion chromatography. MDH activity was assayed in the corresponding column fractions. The elution profile of *M. mazei* chaperonin complexes from the size exclusion column is indicated by the bracket from fractions 7–10. Fractions from the size exclusion chromatography were resolved by 16% SDS-PAGE and immunoblotted against anti-MDH.

**FIG. 8.** Protease protection of GroEL-bound MDH by GroES. GroEL-MDH complexes formed with EcGroEL or MmGroEL as indicated were incubated in Buffer A with or without 0.5 µM AS containing 4 mM AMP-PNP and with respective GroES when indicated, followed by incubation with proteinase K (1.5 µg/ml) for 0–15 min at 25 °C. Proteinase K action was stopped by the addition of 1 mM phenylmethylsulfonyl fluoride. Immunoblot analyses of MDH and the Coomassie Blue staining pattern of GroEL from a representative experiment are shown.

**FIG. 9.** MDH cycling from the trans ring MmGroEL can fold upon addition of ammonium sulfate or upon transfer to the bacterial chaperonin system. MDH refolding was performed under standard assay conditions as described in the legend to Fig. 7A. After 5 min of refolding with 0.5 µM MmGroEL, 1.0 µM MmGroES (●) in Buffer A, 5 mM ATP, either EcGroEL and EcGroES were added at a 5- and 10-fold molar excess to MmGroEL, respectively (○), or 0.5 M AS was added (▴). As a control reaction, the folding of MDH in 0.5 µM MmGroEL, 1.0 µM MmGroES was followed in the presence of 0.5 M AS (○). MDH activity is expressed as a percentage of the activity obtained with 0.5 µM native MDH in Buffer A.
the GroEL-GroES folding cage, suggesting that the *M. mazei* chaperonin has adapted to the slower growth rate of this organism and/or to a subset of slow folding substrate proteins. For the model substrates tested, productive folding was observed only upon encapsulation of the unfolded protein in the *M. mazei* GroEL cavity by GroES. The emergence of only folded proteins from the GroEL/GroES cage may prevent adverse interactions of the GroEL substrates with the thermosome, which is not normally located within the same compartment.

The main functional parameter that differs between the archaecal and the bacterial GroEL/GroES systems is the rate of GroES cycling on and off GroEL. The slower cycling rate of the *M. mazei* system limits the capacity of the chaperonin to release folded protein into the cytosol and this probably explains why *Mm*GroEL/GroES fails to functionally replace the *E. coli* chaperonin, which is adapted to the faster growth rates of this organism. Properties of both *Mm*GroEL and *Mm*GroES contribute to slow cycling. The ATPase activity of *Mm*GroEL is severalfold reduced compared with that of *Ec*GroEL, independently of the stimulating effect of AS. It has frequently been observed that archaeal ATPases are less active than their bacterial counterparts (49, 52–56). A relevant example in this context is the thermosome, whose ATPase is also activated by AS in vitro (49). A comparison of the ATPase centers of *M. mazei* and *E. coli* GroEL based on the structures of *Ec*GroEL and *Ec*GroEL/GroES/ADP (57–60) reveals no obvious differences that may account for the differential ATPase rates, because most critical residues participating in nucleotide binding and hydrolysis are conserved. The only obvious difference is the exchange of alanine 481 in *Ec*GroEL, which contacts the purine ring through van der Waals interactions, by a lysine in *Mm*GroEL. Whether this structural difference is relevant, directly or indirectly, with regard to the stimulatory effect of AS remains to be explored.

*Mm*GroES exhibits an ~15-fold slower dissociation rate from *Mm*GroEL compared with the *E. coli* GroEL/GroES pair, resulting in an increased GroES affinity of *Mm*GroES for GroEL. Interestingly, this effect is also observed upon interaction of *Mm*GroES with *Ec*GroEL and thus reflects, at least in part, a property of GroES independent of the intrinsic ATPase activity of the respective GroEL partner. The association of GroES with GroEL is mediated by flexible loop sequences extending from the base of the GroES oligomer (60–62). Upon binding to GroEL, these sequences assume a β-hairpin structure (60, 63). GroES proteins with loop sequences that are more disordered in solution tend to exhibit a lower affinity for GroEL as a result of the greater entropic penalty incurred upon binding (27, 63).

On the other hand, loop sequences that are more ordered in solution and exhibit higher hydrophobicity in the residues that contact GroEL bind with higher affinity. Indeed, the sequence of the tripeptide in the loop that contacts GroEL directly is more hydrophobic in *Mm*GroES (residues IYI at positions 25–27) than in *Ec*GroES (residues IVL). The tripeptide in *Mm*GroES is similar in hydrophobicity to that in human Hsp10 (IML), which binds more strongly to *Ec*GroEL than to *Ec*GroES (27). Moreover, the highly conserved proline residue in mitochondrial Hsp10 following the hydrophobic tripeptide was shown to reduce the dynamics of the mobile loop, making it less flexible than its bacterial counterpart, which has a threonine at this position (27). The GroES proteins of the *Methanosarcina* species all have a proline after the hydrophobic tripeptide. Additionally, the residue at position 21 in the *Methanosarcina* GroES is a threonine as in mitochondrial Hsp10. This residue was found to increase the affinity of Hsp10 for *Ec*GroEL by increasing the propensity of the sequence to assume a β-hairpin structure in solution (27, 65, 66). *Ec*GroES has a serine at the corresponding position and a serine to threonine mutation in *Ec*GroES results in a higher affinity for GroEL (27).

These features of the *Mm*GroES mobile loop very likely determine the increased affinity for GroEL and may explain why *Mm*GroES restores a functional interaction with the mutant GroEL44 protein, allowing bacterial growth at 43 °C and the propagation of bacteriophages λ and T5 at 37 °C. GroEL44 does not bind appreciably to Gp31, the T4 bacteriophage analog of GroES (27), and also has a drastically reduced affinity for *Ec*GroES. The observation that overexpression of *Mm*GroEL interferes with *E. coli* growth, especially at 43 °C, and with bacteriophage T4 growth at 37 °C is consistent with the possibility that some bacterial substrates and the T4-encoded Gp23 capsid protein may bind the *M. mazei* chaperonin but may not fold in a timely fashion.

Our functional analysis of the *M. mazei* chaperonin system also lends strong support for the significance of protein encapsulation as a major mechanistic feature of chaperonin-assisted folding. The unusual functional dependence of *M. mazei* GroEL/GroES on AS allowed us to compare two modes of action of the chaperonin in vitro, substrate encapsulation in the cis cavity and cycling from the GroEL trans ring. In the absence of AS, the GroEL model substrate MDH went through ATP-dependent cycles of binding and release from the *Mm*GroEL trans ring in a manner non-productive for folding. Productive folding was only achieved upon encapsulation of MDH in the GroEL cis cavity by GroES, and in vitro this step was critically dependent on addition of AS. In contrast, encapsulation of the smaller protein rhodanese occurred independently of AS. We therefore suggest that the presence of AS facilitates the GroES-mediated displacement of MDH from its multiple attachment sites on GroEL into the chaperonin cavity. The fact that *Mm*GroES supports bacteriophage T5 growth on groES619 mutant bacteria and bacteriophage λ and T5 growth on groEL44 mutant bacteria suggests that the *in vivo* environment of the bacterial cytosol can replace the effect exerted by AS in vitro.

Whereas stringent GroEL substrates such as Rubisco and MDH reach the native state rapidly and in a highly efficient manner only through the encapsulation mechanism (64), it was recently shown for *E. coli* GroEL that some of these proteins can fold, albeit slowly and with lower yield, by cycling from the trans GroEL ring when cis encapsulation is disabled (15). The trans mechanism is thought to allow GroEL assisted folding of certain proteins too large to be encapsulated (14). Based on our findings, the *M. mazei* GroEL/GroES system does not support such a reaction, at least under the experimental conditions in which trans cycling was observed. It seems possible that in *M. mazei* the GroEL/GroES system has lost the ability to support trans folding because of the simultaneous presence of the thermosome, which functions independently of a GroES-like cofactor and may be able to assist the folding of larger proteins in a domain-wise manner.

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