Two distantly related classes of cylindrical chaperonin complexes assist in the folding of newly synthesized and stress-denatured proteins in an ATP-dependent manner. Group I chaperonins are thought to be restricted to the cytosol of bacteria and to mitochondria and chloroplasts, whereas the group II chaperonins are found in the archaeal and eukaryotic cytosol. Here we show that members of the archaeal genus Methanosarcina co-express both the complete group I (GroEL/GroES) and group II (thermosome/prefoldin) chaperonin systems in their cytosol. These mesophilic archaea have acquired between 20 and 35% of their genes by lateral gene transfer from bacteria. In Methanosarcina mazei G61, both chaperonins are similarly abundant and are moderately induced under heat stress. The M. mazei GroEL/GroES proteins have the structural features of their bacterial counterparts. The thermosome contains three paralogous subunits, α, β, and γ, which assemble preferentially at a molar ratio of 2:1:1. As shown in vitro, the assembly reaction is dependent on ATP/Mg²⁺ or ADP/Mg²⁺ and the regulatory role of the β subunit. The co-existence of both chaperonin systems in the same cellular compartment suggests the Methanosarcina species as useful model systems in studying the differential substrate specificity of the group I and II chaperonins and in elucidating how newly synthesized proteins are sorted from the ribosome to the proper chaperonin for folding.

The chaperonins are a structurally conserved class of molecular chaperones that assist, in an ATP-dependent manner, in the efficient folding of a subset of newly synthesized and stress-denatured polypeptide chains (1–8). They are found in bacteria, archaea, and eukaryotes and form double-ring toroidal structures with seven to nine subunits of −60 kDa per ring (7). Each ring encloses a central cavity for the binding of a non-native protein. Two classes of chaperonins are distinguished, group I and group II (9–12), which are similar in overall architecture but distantly related in sequence. Group I chaperonins, also known as Cpn60s or Hsp60s, are generally found in the bacterial cytosol (e.g. GroEL in *Escherichia coli*) and in organelles of endosymbiotic origin, such as mitochondria (mtHsp60) and chloroplasts (ribulose-biphosphate carboxylase/oxygenase subunit-binding protein). They cooperate with cofactors of the Cpn10 or Hsp10 family (GroES in *E. coli*). Group II chaperonins occur in the archaeal and eukaryotic cytosol. The archaeal group II chaperonins are commonly referred to as thermosome (Ths), based on the initial description of this complex in the hyperthermophilic archaeon *Pyrococcus furiosus* (13). The eukaryotic group II chaperonin is known as CCT or TRiC (14, 15).

The group I chaperonins (Hsp60s) exist as ~800-kDa homotetradecamers, which are arranged in two stacked heptameric rings. Their Hsp10 cofactors form single homo-heptameric rings that bind to the ends of the Hsp60 cylinder, resulting in the enclosure of protein substrate during folding. The archaeal group II chaperonins form double-ring cylinders with 8- or 9-fold symmetry, consisting of one to three types of homologous subunits. Subunit stoichiometries of 1:1 and 2:1 have been reported for complexes with two types of subunits (13, 16–24). To date two *Sulfolobus* species have been reported to contain three homologous Ths subunits with a stoichiometry of 1:1:1 that form nine-membered rings (25). The eukaryotic group II chaperonin CCT/TRiC has 8-fold symmetry and consists of eight homologous subunits per ring (14, 26). The chaperonins of group II are generally independent of a GroES/Hsp10-like cofactor but functionally cooperate with the GroES unrelated molecular chaperone prefoldin (Pfd; also known as GimC, for factor but functionally cooperate with the GroES unrelated molecular chaperone prefoldin (Pfd; also known as GimC, for gene involved in microtubule biogenesis complex) (27–32). The structural hallmark of all group II chaperonins is a 25-Å-long appendage, which is proposed to function in the enclosure of protein substrate during folding. The archaeal group II chaperonins form double-ring cylinders with 8- or 9-fold symmetry, consisting of one to three types of homologous subunits. Subunit stoichiometries of 1:1 and 2:1 have been reported for complexes with two types of subunits (13, 16–24).

The complete genomes of several mesophilic archaeal species of the genus *Methanosarcina* have recently been sequenced, including those of *Methanosarcina barkeri* (~2.8 Mbp, United States of America; HSE, heat shock element; Hsp, heat shock protein; IPTG, isopropyl-1-thio-beta-D-thiogalactopyranoside; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight; β-ME, β-mercaptoethanol; Mn, M. mazei; MOPS, 4-morpholinopropanesulfonic acid; OD, optical density; Pfd, prefoldin; Ta, *T. acidophilum*; Ths, thermosome; TRiC, TCP-1 containing ring complex.

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$^{*}$ The abbreviations used are: Cpn, chaperonin; CCT, chaperonin containing TCP-1; DTT, dithiothreitol; Ec, *E. coli*; hs, *H. sapiens*; HSE, heat shock element; Hsp, heat shock protein; IPTG, isopropyl-1-thio-beta-D-thiogalactopyranoside; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight; β-ME, β-mercaptoethanol; Mn, *M. mazei*; MOPS, 4-morpholinopropanesulfonic acid; OD, optical density; Pfd, prefoldin; Ta, *T. acidophilum*; Ths, thermosome; TRiC, TCP-1 containing ring complex.

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**Coexistence of Group I and Group II Chaperonins in the Archaeon Methanosarcina mazei***

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Chaperonins in M. mazei

States Department of Energy Joint Genome Institute, Methanosarcina acetivorans (5.8 Mbp) (36), and Methanosarcina mazei G01 (∼4.1 Mbp) (37). A striking feature of the Methanosarcina genomes is that they are the first archaeal genomes identified to contain both group I and group II chaperonin genes, thus adding to the growing evidence of lateral gene transfer between bacteria and archaea (37). How the two groups of chaperonins coevolved remains an intriguing question.

This report describes the molecular cloning, purification, reconstitution, and preliminary functional analysis of both types of chaperonin from M. mazei G01. We demonstrate that both chaperonins are expressed under standard growth conditions of M. mazei and coexist in the cytosolic compartment at a ratio of GroEL to Ths of ∼1:1. Heat stress results in a moderately increased expression. Three Ths subunits, α, β, and γ, were identified, which assemble preferentially at a molar ratio of 2:1:1. In contrast to the α and γ subunits, the β subunit is unable to form homo-oligomeric complexes and thus has a special role in regulating the subunit stoichiometry in the nucleotide-dependent assembly process. Both chaperonins are capable of preventing the aggregation of denatured model substrates, such as mitochondrial rhodanese, but only the GroEL/GroES system supports rhodanese refolding.

EXPERIMENTAL PROCEDURES

Multiple Sequence Alignment

Amino acid sequences were compared using the program Multalin, which creates a multiple sequence alignment from a group of related sequences using progressive pairwise alignments (38, 39).

Analysis of Chaperonins in the Cytosol of M. mazei

Cells of M. mazei G01 were grown at 37 or 45 °C in medium containing 100 mM methanol (40) to an OD660 of 2.0–0.5 (early exponential phase). Cells were harvested by centrifugation at 4 °C for 10 min at 4900 × g. Cell pellets were lyzed in 25 mM MOPS-NaOH, pH 7.5, 5 mM EDTA in the presence of CompleteTM protease inhibitors (Roche Molecular Biochemicals) and 10 μg/ml DNase I by gentle shaking at 4 °C for 1 h. The suspension was centrifuged at 20,800 × g for 20 min at 4 °C to remove cell debris. The supernatant (crude extract) was separated on 10% SDS-PAGE, immunoblotted using the following primers: EcoRI (4–250 bp) and BamHI (1–500 bp) restriction sites. BL21 (DE3) cells (41) were grown and induced (0.25 mM IPTG) as described above. The third construct was grown and induced (0.5 mM IPTG) for 3 h (208,000 ∼ g) at 37 °C after the void volume, whereas the unassembled Mtns subunits appeared at the ~200–330-kDa molecular mass marker. The latter material represented monomeric subunits, as demonstrated by sedimentation velocity and sedimentation equilibrium centrifugation, which followed by sonication (Misonix sonicator, position 4 at 50% duty in pulse mode) and subsequent addition of 0.1 μl/ml benzanilide (250 μg/ml, Merck) to digest DNA and RNA. After removal of cell debris and membranes by centrifugation at 4 °C (20 min, 50,000 × g, followed by 1 h at 100,000 × g), the protein in the supernatant was purified by successive steps of chromatography: DE52 (Whatman) in 30 mM Tris-HCl, pH 7.6, 1 mM DTT, NaCl gradient from 0 to 1 M; Source30Q (Amersham Biosciences) in 25 mM histidine-HCl, pH 5.8, 1 mM EDTA, 1 mM DTT, NaCl gradient from 10 to 500 mM (final pH of loaded sample~6.0); Heparin-HiTrap 5 ml column (Amersham Biosciences) in 30 mM Tris-HCl, pH 7.8, 1 mM DTT, NaCl gradient from 0 to 1 M, and S-300 HR (Amersham Biosciences) in 20 mM MOPS-NaOH, pH 7.4, 100 mM NaCl, 10% glycerol. Appropriate fractions from the final size exclusion column (S-300) were pooled, divided into aliquots, flash-frozen in liquid nitrogen, and stored at −70 °C. Protein concentrations were determined using calculated molar extinction coefficients at 280 nm of 10,360 m−1 cm−1 for MtnGroEL and 5120 m−1 cm−1 for MtnGroES. MtnGroEL and MtnGroES were not detectably contaminated with their respective E. coli homologs, as demonstrated by peptide digests and MALDI-TOF mass spectrometric analysis, consistent with the massive overproduction of the M. mazei chaperonins in E. coli (Fig. 4).

EcGroEL//GroES—E. coli GroEL and GroES were expressed from pet-t11a vectors (Novagen) in BL21 cells and purified as described for MtnGroEL and MtnGroES. Protein concentrations were determined using calculated molar extinction coefficients at 280 nm of 9080 m−1 cm−1 for EcGroEL and 12900 m−1 cm−1 for EcGroES. 

To confirm that the chaperonins exist as oligomeric complexes, crude cell lysates were separated on 10% SDS-PAGE, immunoblotted using the following primers: Mtns α, 5′-CACACTAATAAGGAGATATACATAGGCGAGGAGCACTATATTCTGTTGCC-3′; Mtns β, 5′-CAATATTATTAGAGGTTCCTCTTATAAGTGAAATGTCGCAGCCAGGCAGCAGTTGCTC-3′; and 5′-AGAATTCCGGTATTGAGATTTTACATGCTCTTCATCTGTCGTTGCTGTCGTTGCTGGC-3′. The original archaeal translational TGA stop codon was replaced by TGA for all three subunits. In the case of Mtns γ, it was necessary to replace the original archaeal translational TTG start codon by ATG, allowing for translation in E. coli. The PCR products were inserted into pet-t22b vector (Novagen) using a 5′ NdeI site that includes the translational ATG start codon and a 3′ NheI site just downstream of the translational TGA stop that is followed by a distinct BamHI site. The NheI site in combination with the BamHI site allowed the construction of bi- or polycystronic expression vectors. Because the pet-t22b vector contains a XhoI site downstream from the T7 promoter but upstream of the ribosomal binding site, a Mtns/Ths subunit together with its ribosomal binding site was excised with XhoI and BamHI and inserted into a pet-t22b vector (precut with NdeI and BamHI) containing a translational cassette (for another Mtns/Ths subunit. This procedure resulted in a bicistronic expression vector. The third Mtns subunit was then inserted into the bicistronic expression vector using the same procedure.

BL21(DE3) cells transformed with the respective Mtns-Ths expression constructs were grown and induced (0.25 μM IPTG) as described above. The cells were resuspended in 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, shock-frozen in liquid nitrogen, and stored at −70 °C. Thawed cells were resuspended in lysis buffer (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM EDTA, 5 mM β-mercaptoethanol (β-ME)), supplemented with protease inhibitors (CompleteTM, Roche) and lysozyme (1 mg/ml), incubated at 4 °C for 30 min, followed by sonication as above. The lysate was clarified by centrifugation at 4 °C (208,000 × g) for 30 min. The supernatant was 2-fold diluted with 30 mM Tris-HCl, pH 8.0, 2 mM EDTA, 5 mM β-ME, loaded onto a Source30Q column (220 ml), and eluted with a linear salt gradient (50–500 mM NaCl). Monomeric Mtns/Ths subunits eluted at ~180 mM NaCl and oligomers at ~250 mM NaCl. The relevant fractions were concentrated using Centriprep-30 (Amersham Biosciences) and dialyzed 1:6 with 50 mM Tris-HCl, pH 7.5, 0.5 mM DTT, 2 mM EDTA. This fraction was loaded onto a series of four heparin-Sepharose HiTrap columns (5 ml each) and eluted with 50–1000 mM NaCl. Unassembled subunits eluted at ~250 mM NaCl and the Mtns/Ths oligomers at ~460 mM NaCl. Respective fractions were pooled, concentrated, and loaded onto a Sephacryl S300 HR column (HiLoad 26/60) equilibrated with 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2 mM DTT, and 0.5 M NaCl. This fraction was dialyzed after the void volume, whereas the unassembled Mtns subunits appeared at the ~200–330-kDa molecular mass marker. The latter material represented monomeric subunits, as demonstrated by sedimentation velocity and sedimentation equilibrium centrifugation, which
were performed at 20 °C in a Beckman Coulter™ Optima™ XL-1 analytical ultracentrifuge equipped with absorption optics. The molecular masses were determined using the software Ultracalc 5.0 (B. Demeler, University of Texas, Health Science Center, San Antonio, TX, www.ultrascan.uthscsa.edu). The aberrant fractionation on size exclusion chromatography may be attributed to the elongated shape of chaperonin subunits. The MmThs oligomers were further purified on an anion exchange column (MonoQ 16/1000, Amersham Biosciences) equilibrated with 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM β-ME and eluted with a linear salt gradient (50–500 mM NaCl) at ~280 mM NaCl.

Reconstitution of MmThs Subunits—Assembly of Ths complexes was initiated by incubation of purified monomeric subunits (26.5 ± 1.0 μM Ths α; 13.4 ± 1.0 μM Ths β; 13.4 ± 1.0 μM Ths γ), alone or in different combinations, in assembly buffer (4 mM MOPS-NaOH, pH 7.5, 90 mM NH₄OAc, 9 mM MgCl₂, 4.5 mM ATP or ADP, 16 mM NaCl, 5 mM DTT) at room temperature. Beads were washed as described previously, and supernatant or lysate or lysed E. coli spheroplasts (46) contained assembled subunits, which are highly homologous to each other and to bacterial GroEL and reconstituted MmThs operon. The supernatant was then incubated with protein A-Sepharose beads for 45 min at room temperature. Beads were washed as described previously, and bound proteins were eluted with SDS sample buffer and analyzed by 10% SDS-PAGE, pH 8.8 or 6.5, and immunoblotting with anti-MmThs α and/or β antibodies.

**Prevention of Aggregation and in Vitro Refolding Assays**

Bovine liver mitochondrial rhodanese (Sigma) was denatured in 20 mM MOPS-KOH, pH 7.4, 100 mM KCl, 5 mM MgCl₂, 6 M guanidinium chloride, and 5 mM DTT at a concentration of 50 μM and diluted 100-fold into reactions (20 mM MOPS-KOH, pH 7.4, 100 mM KCl, 5 mM MgCl₂) as specified in the figure legends. Aggregation was followed spectrophotometrically at 320 nm at 30 °C in the presence of different concentrations of chaperonin, as indicated. Rhodanese refolding was performed at 37 °C under the same conditions ± 5 mM ATP, and enzyme activities were determined at 25 °C (42–44). Firefly luciferase (Sigma) was used in aggregation assays as described for rhodanese.

**Antibody Production**

0.5 mg of purified MmGroEL, MmGroES, MmThs α subunit, or MmThs β subunit was injected into rabbits as a 1:1 emulsion with Titer-Max Classic Adjuvant (Sigma) at intervals of 4 weeks, and sera was analyzed for reactivity against M. mazei Göl lysate after 12 weeks using the purified proteins as standards.

**Immunoprecipitations**

Antigenic bands (anti-MmThs α or β) were cross-linked to protein A-Sepharose beads. M. mazei cells were harvested by centrifugation at 4 °C for 30 min at 1000 × g. Cell pellets were lysed by gentle shaking at 4 °C for 30 min in 25 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.01% Tween 20, 2 mM Pefabloc SC (Roche Molecular Biochemicals), and 10 mg/ml DNase I and centrifuged at 20,800 × g for 20 min at 4 °C (45, 46). The M. mazei lysate or lysed E. coli spheroplasts (46) containing recombinant Ths subunits were adjusted to 150 mM NaCl, and incubated with protein A-Sepharose beads for 45 min at room temperature, conditions where Ths complexes remained stable. The supernatant was then incubated with the antibody-cross-linked beads for 1 h at room temperature. Beads were washed as described previously, and bound proteins were eluted with SDS sample buffer and analyzed by 10% SDS-PAGE, pH 8.8 or 6.5, and immunoblotting. 10% SDS-PAGE gels at pH 6.5 resulted in a better separation of MmThs α, β, and γ subunits (see Fig. 5C).

**Mass Determination by Light Scattering**

Ths and GroEL complexes were analyzed by size exclusion chromatography at 37 °C (Toso Haas TSK 4000SW column) with online DAWN EOS multi-angle light scattering (Wyatt Technology, Santa Barbara, CA; 690-nm laser), variable-wavelength UV absorbance at 280 nm (Agilent 1100 series) and Optilab DSP refractive index (Wyatt Technology, Santa Barbara, CA; 690-nm laser) and Optilab DSP refractive index (Wyatt Technology, Santa Barbara, CA; 690-nm laser), variable-wavelength UV absorbance at 280 nm (Agilent 1100 series) and Optilab DSP refractive index (Wyatt Technology, 690 nm) detectors (47, 48). Masses were calculated using the ASTRA software (Wyatt Technology) and a value for dn/dc for protein of 0.185 g/ml.

**Electron Microscopy**

MmGroEL and reconstituted MmThs α, γ complexes were adjusted to a protein concentration of ~50 μg/ml and negatively stained with 2% (w/v) uranyl acetate. Images of MmGroEL and MmThs α, γ complexes were recorded with a CM 20 FEI electron microscope equipped with a 2000 × 2000 CCD camera (nominal magnification, ×47,000). To apply standard correlation averaging methods, the top views of 785 MmGroEL and 345 MmThs α, γ single molecules were extracted from band-pass-filtered images. The average of MmGroEL and reconstituted MmThs α, γ molecules was 7- and 8-fold symmetrized, respectively. The resulting average was then employed as a new reference in a refinement model. To detect interstructural variations, the aligned molecules were subjected to a classification procedure based on eigenvector-eigenvalue data analysis (49).

**Miscellaneous Procedures**

ATPase activities of therosome preparations were determined kinetically for 30 min at 37 °C (50). Denaturing SDS-PAGE analysis was performed according to Laemmli (51). Native polyacrylamide electrophoresis (native PAGE) was performed using 375 mM Tris-HCl, pH 7.5, on 4% gels (52). Western blot analysis with primary polyclonal antibody was carried out in 25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.05% Tween 20, followed by incubation with secondary antibody (horseradish peroxidase-conjugated or alkaline phosphatase-conjugated, anti-rabbit, Sigma) and developed using the ECL luminescence-based or ECF fluor chemistry-based reagents (Amersham Biosciences) (53). Quantitation of blots was performed on a Fuji Phosphorimager using the program Aida (Aida Omega Analyzer version 3.26, Raytest).

**RESULTS**

**Chaperonin Genes in the M. mazei Göl Genome—Analysis of the genome of the mesophilic archaeon M. mazei Göl (Mm) revealed the existence of three conserved genes coding for Ths subunits. The MmThs subunits, termed α, β, and γ, have a molecular mass of 58.9, 58.5, and 58.2 kDa, respectively. As shown for the Ths of Thermoplasma acidophilum (34), the Ths subunits of M. mazei consist of three domains: an equatorial ATP-binding domain, an intermediate hingelike domain, and an apical domain containing the binding site for protein substrate. A sequence alignment of the group II chaperonins of Methanosarcina species with their respective archaeal and eukaryotic homologs showed homologies of 50–80%. Although a sequence comparison of the apical domains of the MmThs α and γ subunits revealed ~70% identity and ~85% similarity, the β subunit interestingly shows only ~35% identity/~60% similarity to other α or γ (Fig. 1A). The apical domain of the MmThs α subunit is ~50–95% identical and ~80–97% similar to the apical domain of Ths α subunits of other archaeal species and ~29% identical/~60% similar to the TCP-I subunit of the eukaryotic group II chaperonin TRC/CCT (Fig. 1A). Analysis of the helical protrusions of the apical domain again showed that the MmThs β subunit has only a relatively low degree of homology (30–45% identity/60–70% similarity) to the MmThs α or γ subunits, which are highly homologous to each other and to the Ths α orthologs of other archaeal species (~70% identity/~80% similarity) (Fig. 1A). We also identified two genes in the M. mazei genome encoding the subunits of the potential Ths cofactor Pfd, which is known to assist in group II chaperonin-mediated protein folding in the eukaryotic cytosol (27–31, 55). Pfd of Methanobacterium thermoautotrophicum is a hexameric complex of ~84.6 kDa consisting of two central α-type subunits and four peripheral β-type subunits (56). The α and β subunits of MmPfd are highly homologous to the α and β subunits of M. thermoautotrophicum Pfd (MmPfd) (Fig. 1B) (27). Analysis of the M. mazei genome also revealed the presence of a groE operon encoding the group I chaperonin MmGroEL and its cofactor MmGroES (Fig. 2A) (37). The genome sequences of two other archaea of the genus Methanosarcina, M.arkeri fusaro (United States Department of Energy Joint Genome Institute) and M. acetivorans (36), also contain the groE operon. The general structure of the groE operon of M. mazei is identical to that of the bacterial groE operon from E. coli (57). However, the groE operon of M. mazei has
a putative promoter with high homology to the archaeal pro-
moter consensus sequence AAANNTTTATATA (58, 59) and the
archaeal heat shock element (HSE) consensus sequence CCGA-
promoter-GAA (60). Furthermore, the 3′/H11032
end of the
MmgroESL
operon contains an oligo(T)-rich region that is also character-
istic of archaeal termination sites (Fig. 2
A
) (59).

A comparison of the three
Methanosarcina
GroEL sequences
with group I chaperonin homologues of bacteria (E. coli), mito-
chondria (human; hs) and chloroplasts (Arabidopsis thaliana)
shows a high homology in the apical domains of GroEL with at
least 47% identity and 65% similarity (Fig. 2
B
), whereas the
Methanosarcina
GroEL sequences themselves share 81% iden-
tical residues (85% similarity). Analysis of the bacterial, hu-
man, and plant GroES sequences indicated
FIG.1 .

The group II chaperonin system of
M. mazei.
A, sequence alignment of the apical domains of thermosome proteins: M. mazei
(METMAα (NP_633403), METMAβ (NP_632096), METMAγ (NP_633120)); M. barbieri (METBAα: Contig1921, United States Department of
Energy Joint Institute); M. acetivorans (METACα (NP_615060)); T. acidophilum (THEACα; 026320; Refs. 18 and 34); M. thermoautotrophicum
(METTHα; 027646 and 026774). Box indicates the amino acids forming the helical protrusion (33, 34). Secondary structure elements are indicated as rectangles for α-helices and arrows for β-sheets with reference to the structure of the isolated apical domain from T. acidophilum (33). Note that in the structure of the T. acidophilum complex (34), amino acids 245–247 and 261–266 assume β-sheet structure (underlined).
B, alignment of
prefoldin
α and
β subunits of
M. mazei
(METMA (NP_632833 and NP_632698) and
M. thermoautotrophicum
(METTH (NP_276721 and
NP_275820)). Numbers in parentheses refer to the respective GenBank™ accession numbers. Highly conserved residues are shown in red, less conserved residues are in blue. The secondary structure elements are indicated as rectangles for α-helices and arrows for β-sheets with reference to the structure of M. thermoautotrophicum (56). Green arrows indicate the inserted β-hairpin structure in the prefoldin α subunits, which mediates the assembly of the central α subunit dimer of the prefoldin complex.
~56% similarity, with ~91% identity and ~97% similarity within the three Methanosarcina species. Interestingly, the mobile loop sequence of MmGroES that mediates the interaction with GroEL shows higher homology to the corresponding sequence of human mitochondrial Hsp60 than to EcGroES. In both MmGroES and hsHsp10 the hydrophobic tripeptide sequence IYI (residues 25–27 in MmGroES) and IML (residues 31–33 in hsHsp10), which makes direct contact with GroEL (61, 62), is followed by a proline (Fig. 2C), whereas EcGroES has a threonine in this position. The proline is postulated to reduce the flexibility of the mobile loop sequence for free GroES (63), thus entropically favoring the GroEL-bound state of GroES (63–65).

Both Chaperonins and Their Cofactors Are Expressed as Oligomeric Proteins and Are Induced by Heat Stress—To determine whether all the M. mazei chaperonin genes are expressed at the protein level, we initially analyzed cell lysates of M. mazei by SDS-PAGE and immunoblotting with antibodies directed against E. coli GroEL and the α subunit of T. acidophilum Ths (TuThs). Both antibodies detected proteins of the expected sizes (Fig. 3A, lanes 1 and 3). Additionally, the cofactors GroES and Pfd were also detected with antibodies against
E. coli GroES and the Mt Pfd α subunit, respectively (Fig. 3A, lanes 2 and 4). Subsequent analysis was performed with antibodies raised against the recombinant M. mazei proteins. Size exclusion chromatography followed by immunoblotting revealed that the respective proteins form oligomeric complexes (Fig. 3B) that fractionate essentially like their known homologs. Note that, because of its unusual shape, the ~84.6-kDa MmPfd complex migrates at ~160 kDa upon size exclusion chromatography (27, 56).

To allow for a direct comparison of the expression levels of both chaperonins, the high molecular weight fractions of the cell lysate (Fig. 3B, fractions 22–28) were further analyzed by native PAGE (Fig. 3C). The complexes corresponding to MmGroEL and MmGroES by immunoblotting were clearly stained with Coomassie Blue and exhibited the typical migration properties of Ths and GroEL on native PAGE (24). MmGroEL and MmGroES were of similar abundance by Coomassie staining and quantitative immunoblotting with purified MmThs α and MmGroEL as standards (0.5–1% of cytosolic protein; data not shown). Assuming a total protein concentration of 200 g/liter in the cytosol, the cellular oligomer concentration of MmGroEL and MmThs was estimated at ~1–2 μM. A third abundant high molecular weight complex in the M. mazei cytosol with a characteristic migration behavior is the proteosome (Fig. 3C).

Fig. 3. Coexpression of group I and II chaperonins and their co-factors in the cytosol of M. mazei. A, immunoblot analysis of a 20,800 × g supernatant of M. mazei cell lysate with antibodies against E. coli (Ec) GroEL and GroES, T. acidophilum (Ta) thermosome α subunit, and the α subunit of M. thermoautotrophicum (Mt) Pfd. B, fractionation of M. mazei (Mm) lysate (20,800 × g supernatant) on a Superdex S-200 2.3/30 size exclusion column followed by 15% SDS-PAGE and immunoblotting with antisera against recombinant M. mazei GroEL, GroES, Ths α subunit, and Pfd α subunit. The positions of molecular weight standards are indicated. C, analysis of the combined fractions 20–28 from B by 4.5% native PAGE and immunoblotting with antisera against recombinant M. mazei GroEL and Ths α subunit. D, lysates from M. mazei cells exposed to heat stress at 45 °C (shift from 37 °C) for 0–30 min were analyzed by SDS-PAGE and immunoblotting with the antisera against M. mazei GroEL and Ths α as in B. Quantitation of immunoblots from three independent experiments is shown in the right panel.

Consistent with the presence of a putative HSE in the chaperonin genes (60, 66, 67), the levels of both MmGroEL and MmThs chaperonins increased when M. mazei cells were exposed to heat stress at 45 °C for up to 30 min, as revealed by immunoblotting of cytosolic extracts with antibodies against MmThs α and MmGroEL. Quantitative analysis showed that the expression levels of both chaperonin subunits increased moderately by 2–2.5-fold upon temperature shift from 37 to 45 °C (Fig. 3D).

Characterization of Recombinant M. mazei Chaperonins—MmGroEL and MmGroES were cloned and expressed in E. coli. Both proteins were highly overexpressed to levels 30–50-fold above those of the endogenous E. coli homologs (Fig. 4A). As expected, MmGroEL and MmGroES were produced as oligomers of ~800 and ~70 kDa, respectively, as demonstrated by size exclusion chromatography and multi-angle light scattering (see below and data not shown). Peptide digests followed by...
MALDI-TOF mass spectroscopy confirmed the identity of the purified M. mazei proteins but failed to detect the E. coli homologs, thus excluding a functionally relevant contamination with host GroEL and GroES.

The MmThs α, β, and γ genes were also cloned and expressed in E. coli either separately or in various combinations under control of the T7 promoter. In all cases large amounts of soluble protein were produced after induction with IPTG (Fig. 4, B and C). MmThs complexes and incompletely assembled subunits were purified by a combination of ion exchange and size exclusion chromatography. MmThs complexes eluted from a Sephacryl S-300 HR size exclusion column at ~900 kDa, whereas incompletely assembled subunits eluted at 200 kDa (for β) and at 330 kDa (for α and γ). The latter proteins behaved predominantly as monomers by analytical ultracentrifugation (data not shown). Interestingly, the MmThs β subunit did not form a ~900-kDa complex but fractionated exclusively as the monomer.

As shown previously, the two groups of chaperonin have a unique migration behavior on native PAGE, with the archaeal and eukaryotic group II chaperonins migrating more slowly than GroEL (24). This behavior was also noted for the MmThs obtained from subunits recombinantly expressed in E. coli and for recombinant MmGroEL and MmGroES (data not shown). When E. coli cell lysates containing overexpressed M. mazei Ths subunits were analyzed by native PAGE (Fig. 4C), the α and γ MmThs subunits migrated as distinct high molecular weight complexes, very similar to bovine TRIC (Fig. 4C, lanes 1, 2, and 4). In contrast, a different migration behavior was noted for the MmThs β subunit, which, on its own, does not form a high molecular weight complex, as stated above. The slow migration of un assembled β subunit on native PAGE (Fig. 4C, lane 3) may be the result of the high isoelectric point of the protein of 5.49 (compared with 4.59 for the α subunit and 4.81 for the γ subunit) (see also Fig. 6A). Interestingly, when the β subunit was coexpressed with α or γ subunit, the formation of distinct high molecular weight complexes on native PAGE, seen with α and γ alone, was no longer observed in the case of α or was significantly reduced in the case of γ (Fig. 4B, lanes 5 and 7). As shown in Table I, complexes of the β subunit with either the α or γ subunit can be observed by size exclusion chromatography but are unstable under the conditions of native PAGE. In contrast, when the α, γ or α, β, γ subunits were coexpressed in E. coli, distinct high molecular weight complexes were observed by native PAGE (Fig. 4C, lanes 6 and 8). These results suggested that complex subunit interactions regulate the oligomeric assembly of Ths subunits and raised the question as to the subunit composition of the Ths complex(es) present in M. mazei cells in vivo. A 15% SDS-PAGE gel of purified MmGroEL and MmGroES together with the purified MmThs subunits is shown in Fig. 4D.

### Table I

**Subunit composition of in vitro assembled thermosome complexes**

| Input ratio | Ratio in HMW complex |
|-------------|----------------------|
| α          | All-α                |
| β          | No HMW complex       |
| γ          | All-γ                |
| α:β        | 1:0.5                | 1:0.58        |
| α:β        | 1:1                  | 1:0.82        |
| α:γ        | 1:0.5                | 1:0.57        |
| α:γ        | 2:1                  | 1:0.95        |
| γ:β        | 1:0.5                | 1:0.44        |
| γ:β        | 2:1                  | 2:1.13        |
| α:β:γ      | 2:0.5:1              | 2:1.2:1.3     |

*Fig. 4. Recombinant expression of M. mazei chaperonins in E. coli.* A, soluble fractions from E. coli cells overexpressing MmGroEL or MmGroES subunits were analyzed by 15% SDS-PAGE and Coomassie staining. Arrowheads point to the positions of MmGroEL and MmGroES, respectively. B, soluble fractions were prepared from E. coli cells expressing the MmThs subunits indicated and analyzed by 15% SDS-PAGE and Coomassie staining. Positions of molecular size markers are indicated in kDa. C, analysis of soluble fractions from β and of purified recombinant E. coli GroEL and MmGroEL by native PAGE (4%) and Coomassie staining. D, purified recombinant M. mazei Ths, GroEL, and GroES proteins (15% SDS-PAGE). Positions of molecular size markers are indicated in kDa.
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Subunit Composition of Endogenous M. mazei Thermosome Complexes—To determine the subunit composition of the endogenous MmThs chaperonin complex(es) by co-immunoprecipitation experiments, antibodies were raised in rabbits against the recombinant Ths α and β subunits, i.e. those subunits with the lowest degree of sequence homology. Characterization of these antibodies by immunoblot analysis showed that the anti-Ths α antibody reacted strongly with purified recombinant α subunit and exhibited only a weak (−10-fold lower) cross-reactivity with the γ subunit (Fig. 5A, left panel). The anti-MmThs β antibody was monospecific for the β subunit (Fig. 5A, right panel). The two antibodies were further characterized by immunoprecipitation experiments with E. coli lysates from cells expressing either recombinant MmThs all-α or all-γ complex, or soluble, unassembled β subunit (Fig. 5B). Analysis of the immunoprecipitates by immunoblotting with a mixture of the antibodies demonstrated that both the anti-α and the anti-β antibody are subunit-specific under native conditions. There was no cross-reactivity with MmGroEL (data not shown).

Using these antibodies, we first analyzed the fractions of a size exclusion chromatogram of a total soluble extract of M. mazei cells for the presence of the three subunits (Fig. 5C). All three Ths subunits were exclusively recovered in the size range corresponding to the chaperonin complex by immunoblotting and thus do not detectably exist as free subunits. Quantitative analysis with the recombinant α, β, and γ subunits as standards indicated that the proteins occur in vivo at a molar ratio of α:β:γ of ∼2:1:1. Co-precipitation experiments were performed from M. mazei cell extracts to determine whether the three subunits co-assemble into hetero-oligomeric complexes or form separate homo-oligomeric assemblies. The anti-α antibody clearly precipitated all three subunits (Fig. 5D). Again, quantitative immunoblotting revealed a molar ratio of α:β:γ of ∼2:1:1. Likewise, the anti-β antibody also precipitated all three subunits in the same ratio (Fig. 5D). Thus, although the co-existence of complexes with different subunit compositions cannot be ruled out, the most plausible conclusion from these results is that the majority of Ths subunits populate a defined hetero-oligomeric chaperonin complex containing two molecules of α per molecule of β and γ subunit. Such a complex would be expected to have 8-fold symmetry with 4 α, 2 β, and 2 γ subunits per ring. Unfortunately, efforts to determine the symmetry of the purified endogenous chaperonin complex by electron microscopy failed, as a result of the instability of the complex under the conditions of negative stain electron microscopy.

Nucleotide-dependent Assembly of M. mazei Thermosome Subunits—To obtain more insight into the co-assembly properties of the MmThs subunits, we studied their reconstitution into chaperonin complexes in vitro from the recombinant, monomeric proteins. On native PAGE the monomeric MmThs α and γ subunits migrated much faster than the oligomeric complexes observed in cell lysates (Fig. 6, lanes 1–3 and 7). In contrast, the unassembled β subunit migrated aberrantly slowly (Fig. 6, lane 5), as noted above (Fig. 4). Upon incubation in the presence of ATP and MgCl₂, nearly the entire population of α and γ subunits assembled into the respective, slowly migrating oligomeric complexes (Fig. 6, lanes 1–4, 7, and 8). (Note that the subunits differ in their staining intensity with Coomassie Blue upon complex formation.) The same effect was observed with ADP and MgCl₂ (data not shown), indicating that assembly depends on nucleotide binding, not hydrolysis. In contrast, the monomeric β subunit did not change its migration behavior in the presence of nucleotide and Mg²⁺ (Fig. 6, lanes 5 and 6), consistent with its inability to form stable, homo-oligomeric complexes as observed upon expression in E. coli (see Fig. 4). Interestingly, when β subunits were mixed with α subunits at a 1:1 molar ratio and incubated with ATP/ Mg²⁺, the all-α complex was only barely detectable and unassembled α subunit accumulated (Fig. 6, lane 9). On the other hand, the 1:1 combination of α and γ subunits resulted in the efficient reconstitution of a complex that migrated between the all-α and all-γ oligomers and apparently consisted of both subunits (Fig. 6, lane 10). The incubation of β and γ subunits resulted in the formation of a complex that migrated similar to the αγ complex (Fig. 6, lane 11), in agreement with the results obtained upon coexpression of β and γ subunits in E. coli (see Fig. 4B, lane 7). The 1:1:1 combination of α, β, and γ subunits gave rise to a MmThs complex(es) that migrated slightly faster than the all-α complex (Fig. 6, lane 12). Complex formation consumed the majority of α and γ subunits but only about half...
of the amount of β subunits present in the reaction.

The special role of the β subunit in the assembly process was examined further by incubating the Ths subunits at different ratios in the presence of ATP/Mg²⁺, followed by analysis of the complexes by size exclusion chromatography and Western blotting instead of native PAGE (Table I). Although confirming the inability of the β subunit to form homo-oligomers, these experiments revealed the capacity of β to form a complex with α at a stoichiometry of α:β of −1:0.5. Thus, we conclude that the interaction of α with β is relatively unstable and therefore not detectable by native PAGE but is stabilized in the additional presence of the γ subunit (see Fig. 6). Interestingly, the 1:0.5 composition of the αβ complex, observed by size exclusion chromatography, was also obtained at an input ratio of αβ subunits of 1:1 (Table I), with half of the total β subunit fractionating as the monomer (data not shown). In contrast, with all other binary subunit combinations, the composition of the resulting complexes reflected the input ratio of subunits (Table I). Thus, in contrast to γ the β subunit is unable to form a 1:1 complex with α, a property that presumably governs the overall assembly process. In support of this conclusion, whether the three subunits were incubated at a ratio of αβ:γ of 2:0:5:1 or 2:1:1, the resulting complex contained the subunits at the 2:1:1 ratio.

Negative stain electron microscopy and averaging of ~350 images demonstrated that the reconstituted MmThs αγ complex has an 8-fold symmetrical ring structure with a diameter of ~16.7 nm (Fig. 7A), in close agreement with the 8-fold symmetrical Ths complex of T. acidophilum (18). Side views of the MmThs αγ complex showing double rings were observed but not in sufficient number for averaging. For the Ths complex isolated from M. mazei cells and the complex reconstituted from α, β, and γ subunits, negative strain images of sufficient quality could not be obtained. Nevertheless, these Ths complexes must also have double-ring structures, based on their typical fractionation by size exclusion chromatography and native PAGE, and their mass of 925 ± 17 kDa determined by multi-angle light scattering (data not shown). MmGroEL showed the expected 7-fold symmetry with a diameter of ~14.7 nm (Fig. 7B) (52) and a mass of 794 ± 14 kDa by multi-angle light scattering (data not shown).

Functional Properties of the M. mazei Chaperonins—E. coli GroEL has been shown to prevent the aggregation of several model polypeptides upon their dilution from denaturant into refolding buffer (27, 42, 68). We used mitochondrial rhodanese (33 kDa) and firefly luciferase (62 kDa) as aggregation prone test substrates. Aggregation was followed spectrophotometrically by measuring the turbidity of the solution at 320 nm. GroEL has been shown to prevent the aggregation of several model polypeptides upon their dilution from denaturant into refolding buffer (27, 42, 68). We used mitochondrial rhodanese (33 kDa) and firefly luciferase (62 kDa) as aggregation prone test substrates. Aggregation was followed spectrophotometrically by measuring the turbidity of the solution at 320 nm. GroEL was run as a standard (lanes 13).
dependent on MmGroEL, MmGroES, and ATP and with the same efficiency as with EcGroEL/GroES (Fig. 9). MmGroEL/GroES did not mediate the refolding of firefly luciferase (data not shown), again in agreement with observations made with the E. coli chaperonin (15). Luciferase slightly exceeds the size limitation of the GroEL/GroES cage, and this may explain why its interaction with GroEL/GroES is non-productive for folding.

The reconstituted MmThs αβγ was inactive in the refolding assay with both rhodanese and luciferase (data not shown). The failure of MmThs to refold these heterologous model proteins could have been the result of a lack of ATPase activity of the chaperonin. To address this possibility, we measured the ATPase activity of the various reconstituted Ths preparations described above. The reconstituted MmThs αβγ showed the highest ATPase activity under normal buffer conditions, followed by the all-α Ths and the αγ complex (Table II). Interestingly, unassembled β subunit and the all-γ Ths had either no detectable or very low ATPase activity (Table II). Thus, in the αγ and αβγ complexes, the activity of the α subunit is either very strongly stimulated or, more likely, the ATPase inactive β and γ subunits acquire significant ATP hydrolytic activity in the oligomeric context with α subunits. As described for Ths complexes from methanogenic archaea (69), the ATPase of the

| Table II | ATPase activity of reconstituted MmThs complexes |
|----------|-----------------------------------------------|
| Reconstituted MmThs | (NH₄)₂SO₄ |
| α | 2.5 | 8.2 |
| β | ND | ND |
| γ | <0.1 | <0.1 |
| αγ | 2.3 | 6.6 |
| αβγ | 4.7 | 8.2 |

FIG. 8. Aggregation prevention activity of MmGroEL and MmThs. Aggregation of denatured mitochondrial rhodanese (A and C) and firefly luciferase (B and D) upon dilution from denaturant was monitored spectrophotometrically in the presence of MmGroEL (A and B) and reconstituted MmThs αβγ (C and D), as described under “Experimental Procedures.” Final concentrations of rhodanese and luciferase were 0.5 μM, and the chaperonin concentrations used are indicated.

FIG. 9. Refolding of rhodanese mediated by MmGroEL/GroES. Rhodanese refolding assays were performed under the conditions described in Fig. 8 at 37 °C in the absence of chaperonin (●); presence of 0.5 μM MmGroEL, 5 mM ATP (●); 0.5 μM MmGroEL, 1 μM MmGroES, 5 mM ATP (○); or 0.5 μM EcGroEL, 1 μM EcGroES, 5 mM ATP (●). At the times indicated, chaperonin activity was inhibited by the addition of CDTA and rhodanese activity determined at 25 °C. 100% corresponds to native enzyme control.

MmThs αβγ was inactive in the refolding assay with both rhodanese and luciferase (data not shown). The failure of MmThs to refold these heterologous model proteins could have been the result of a lack of ATPase activity of the chaperonin. To address this possibility, we measured the ATPase activity of the various reconstituted Ths preparations described above. The reconstituted MmThs αβγ showed the highest ATPase activity under normal buffer conditions, followed by the all-α Ths and the αγ complex (Table II). Interestingly, unassembled β subunit and the all-γ Ths had either no detectable or very low ATPase activity (Table II). Thus, in the αγ and αβγ complexes, the activity of the α subunit is either very strongly stimulated or, more likely, the ATPase inactive β and γ subunits acquire significant ATP hydrolytic activity in the oligomeric context with α subunits. As described for Ths complexes from methanogenic archaea (69), the ATPase of the
all-α, αγ, and αβγ complexes was activated between 2- and 4-fold in the presence of 200 mM ammonium sulfate (Table II). Thus, MnTThs complexes analyzed fulfill several criteria of functionality, suggesting that the inability to refold heterologous model proteins reflects a specialization for certain M. mazei substrates, which remain to be identified.

**DISCUSSION**

Using M. mazei as an example, we have shown here that the archaea of the genus *Methanosarcina* coexpress both the complete group I (GroEL/GroES) and group II (thermosome/prefoldin) chaperonin systems in the cytosol under normal growth conditions. The group I chaperonins have previously been thought to be restricted to the bacterial cytosol and to mitochondria and chloroplasts. The MnGroEL/GroES system exhibits the structural and functional features of its bacterial homologue. The MnTThs contains three homologous subunits, of which the β subunit has a critical role in regulating the nucleotide/Mg$^{2+}$-dependent assembly process, resulting in the preferential formation of a complex consisting of α, β, and γ subunits at a molar ratio of 2:1:1. This complex exhibits optimal ATPase activity. The co-existence of both chaperonin systems in the same cellular compartment suggests that the *Methanosarcina* should become useful in studying how newly synthesized proteins are sorted to their respective chaperonin and in elucidating the substrate specificity of the group I and II chaperonins.

The *Methanosarcina* are metabolically diverse methanogens that can utilize methanogenic substrates, such as acetate, methyamines, and methanol. Their unique capability among the archaea to form complex multicellular structures during different phases of growth and in response to environmental changes enables them to colonize diverse environments (66). Three genome sequencing projects have suggested that the *Methanosarcina* species have acquired 20–85% of their genes by lateral gene transfer from bacteria, and this likely explains the presence of both the group I and group II chaperonin systems in these organisms. Unlike many other archaea that lack the Hsp70 chaperone system (70, 71), the *Methanosarcina* also contain homologues of bacterial DnaK (Hsp70) and its cofactors DnaJ and GroE (67, 72), in addition to the two chaperonin systems. Bacterial trigger factor, on the other hand, a chaperon with peptidyl prolyl cis/trans isomerization activity that binds to nascent polypeptide chains and is partially functionally redundant with DnaK (45, 73), is absent in M. mazei and all other archaea analyzed to date (7).

GroEL and the thermosome are among the major molecular weight protein complexes detectable in the M. mazei cytosol by native PAGE and size exclusion chromatography. The two chaperonins occur at roughly equimolar concentration and are both moderately heat-stress-inducible. In *E. coli*, approximately 10–15% of newly synthesized polypeptides transit GroEL during folding (46, 74). The fraction of archaean proteins interacting with the thermosome has not yet been determined, but the eukaryotic group II chaperonin TRiC/CCT in mammalian cells interacts with 9–15% by mass of newly synthesized chains (75), with actin and tubulins being the predominant substrates. The co-occurrence of both types of chaperonin in the *Methanosarcina* species suggests that each system may assist in the folding of a subset of cytosolic proteins, with GroEL/GroES likely being essential for the folding of some of the proteins of bacterial origin. It is also possible that the presence of GroEL/GroES has facilitated the specialization of the M. mazei thermosome in the folding of proteins that cannot be handled by GroEL. By analogy with the eukaryotic system, prefoldin may function as a co-chaperon in specific substrate delivery to the thermosome (28, 32). Analysis of the protein flux through the *Methanosarcina* chaperonin systems now offers a unique opportunity to understand the differential substrate specificity of group I and group II chaperonins.

Phylogenetic analyses of archaeal chaperonins have revealed a complex pattern of lineage-specific gene duplications and gene losses, suggesting that hetero-oligomeric chaperonin complexes have arisen, and been lost, multiple times in the evolutionary history of this group (25, 76, 77). Unlike most other archaeal species analyzed, the *Methanosarcina* have three homologous Ths subunits that differ most in their apical domain sequences, including their α-helical protrusions as the likely sites of substrate recognition (33, 35, 78). Our analysis of the subunit composition of the endogenous Ths complex by quantitative immunoblotting and co-immunoprecipitation in conjunction with the *in vitro* analysis of the assembly process suggests that the chaperonin complex in *M. mazei* contains 4:2 α, 2 β, and 2 γ subunits per 8-membered ring, which may be arranged as αβ αγ αγ or αα βγ αα βγ. The latter possibility appears more likely because, in contrast to all other subunit compositions, the α and β subunits alone will only form a 2:1 complex. The structural basis for the special nature of the αβ subunit interaction remains to be explored. We note, however, that at this point the co-existence of 2:1:1 αβγ Ths complexes with complexes of different subunit composition and symmetry cannot be ruled out. An 8-fold symmetrical, alternating arrangement of α and β subunits has been reported for the Ths of *T. acidophilum* (34, 79), whereas 9-fold symmetrical rings have been seen for the three-subunit Ths of two *Sulfolobus* species (25).

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