MtGimC, a novel archaeal chaperone related to the eukaryotic chaperonin cofactor GimC/prefoldin

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Group II chaperonins in the eukaryotic and archaeal cytosol assist in protein folding independently of the GroES-like cofactors of eubacterial group I chaperonins. Recently, the eukaryotic chaperonin was shown to cooperate with the hetero-oligomeric protein complex GimC (prefoldin) in folding actin and tubulins. Here we report the characterization of the first archaeal homologue of GimC, from *Methanobacterium thermoautotrophicum*. MtGimC is a hexamer of 87 kDa, consisting of two α and four β subunits of high α -helical content that are predicted to contain extended coiled coils and represent two evolutionarily conserved classes of Gim subunits. Reconstitution experiments with MtGimC suggest that two subunits of the α class (archaeal Gim α and eukaryotic Gim2 and 5) form a dimer onto which four subunits of the β class (archaeal Gim β and eukaryotic Gim1, 3, 4 and 6) assemble. MtGim α and β can form hetero-complexes with yeast Gim subunits and MtGim β partially complements yeast strains lacking Gim1 and 4. MtGimC is a molecular chaperone capable of stabilizing a range of non-native proteins and releasing them for subsequent chaperonin-assisted folding. In light of the absence of Hsp70 chaperones in many archaea, GimC may fulfil an ATP-independent, Hsp70-like function in archaeal *de novo* protein folding.

Keywords: chaperonin/GimC/prefoldin/protein folding

Introduction

A significant fraction of newly synthesized polypeptides depend on molecular chaperones for their efficient folding (Ellis, 1993; Georgopoulos and Welch, 1993; Hartl, 1996; Johnson and Craig, 1997; Netzer and Hartl, 1998). Chaperones of the Hsp70 family recognize extended hydrophobic peptide segments exposed by non-native

polypeptides, and prevent nascent polypeptide chains on ribosomes from misfolding and aggregating (Beckmann *et al.*, 1990; Pfund *et al.*, 1998; Deuerling *et al.*, 1999; Teter *et al.*, 1999; Thulasiraman *et al.*, 1999). In *Escherichia coli*, Hsp70 cooperates with the chaperone trigger factor in protecting nascent chains (Deuerling *et al.*, 1999; Teter *et al.*, 1999). Whereas most proteins in the cytosol probably fold rapidly upon completion of synthesis and release from nascent chain-binding chaperones, a subset of slow folding and aggregation-prone polypeptides depends on further assistance by a chaperonin for efficient folding. In *E.coli*, ~10% of newly synthesized polypeptides transit the chaperonin GroEL (Horwich *et al.*, 1993; Ewalt *et al.*, 1997). The eukaryotic chaperonin TRiC (also termed CCT) is required for the folding of tubulins and actin and a number of additional proteins (Sternlicht *et al.*, 1993; Kubota *et al.*, 1995; Hynes *et al.*, 1996; Lewis *et al.*, 1996; Thulasiraman *et al.*, 1999). Hsp70 and chaperonin systems are ubiquitous in bacteria and in the cytosol and organelles of eukaryotes, and have been proposed to cooperate in chaperone-assisted protein folding pathways (Langer *et al.*, 1992; Buchberger *et al.*, 1996; Hartl, 1996; Teter *et al.*, 1999). The absence of Hsp70 homologues in numerous archaea (Gribaldo *et al.*, 1999) is therefore unexpected (Macario and de Macario, 1999) and suggests that an unknown molecular chaperone(s) may perform Hsp70-like functions during the *de novo* folding of some cytosolic proteins.

The chaperonins are cylindrical complexes of 800–1000 kDa and are composed of two rings with 7–9 subunits each (Willison and Horwich, 1996; Bukau and Horwich, 1998; Ranson *et al.*, 1998). They capture non-native polypeptides in their central cavity via hydrophobic binding surfaces. In the case of group I chaperonins of eubacteria, mitochondria and chloroplasts, ATP-dependent capping of the polypeptide-containing ring by the dome-shaped cofactor GroES causes the displacement of the bound polypeptide into an enclosed cage in which it is free to fold but unable to associate non-productively with other non-native polypeptides (Mayhew *et al.*, 1996; Weissman *et al.*, 1996). Given the close cooperation between GroEL and GroES during protein folding, it is intriguing that the group II chaperonins from the cytosol of eukaryotes (TRiC or CCT) and archaea (thermosome), both of which have overall structures that are very similar to group I chaperonins (Braig *et al.*, 1994; Rosemann *et al.*, 1996; Ditzel *et al.*, 1998; Llorca *et al.*, 1999), function without a GroES-like cofactor. While it is proposed that the thermosome and TRiC have a built-in capacity to open and close their central cavities via extensions of their apical domains (Klumpp *et al.*, 1997; Ditzel *et al.*, 1998; Nitsch *et al.*, 1998; Llorca *et al.*, 1999), there is also recent evidence that *in vivo* and *in vitro* TRiC functions jointly with a hetero-oligomeric complex

termed GimC or prefoldin (Geissler *et al.*, 1998; Vainberg *et al.*, 1998; Hansen *et al.*, 1999; Siegers *et al.*, 1999).

Little is known about the structure and function of GimC. Both yeast GimC and bovine prefoldin (PFD) contain six distinct, evolutionarily conserved subunits, compared with eight for the cytosolic chaperonin TRiC. The exact subunit composition and structural arrangement of the complex remain to be determined. GimC/PFD interacts with TRiC *in vivo* as well as *in vitro* (Vainberg *et al.*, 1998; Siegers *et al.*, 1999). Consistent with the participation of TRiC and GimC/PFD in shared protein folding pathways, disruption of *GIM* genes in yeast results in actin and tubulin cytoskeletal phenotypes that are essentially identical to those of various TRiC subunit mutants (Stoldt *et al.*, 1996; Geissler *et al.*, 1998; Vainberg *et al.*, 1998; Siegers *et al.*, 1999). In yeast strains deleted of one or more *GIM* genes, the folding of actin by TRiC is delayed ~5-fold relative to wild-type cells. This reduced rate of folding is correlated to a decreased rate of actin release from TRiC and to an increased leakage of non-native forms of actin into the cytosol (Siegers *et al.*, 1999). Interestingly, GimC/PFD can bind denatured forms of actin and tubulin, suggesting that it plays a more active role during protein folding than does GroES, which does not interact with non-native polypeptides. However, the association of GimC/PFD with TRiC appears to be more transient than the interaction of GroES with GroEL, perhaps reflecting the existence of dynamic ternary complexes during protein folding for the eukaryotic chaperonin system. Both GimC and TRiC may bind to their substrates late in translation (Frydman *et al.*, 1994; Hansen *et al.*, 1999) and a role for GimC/PFD in targeting polypeptides to TRiC has been suggested (Vainberg *et al.*, 1998; Hansen *et al.*, 1999). On the other hand, functional GimC is not essential for actin binding to TRiC *in vivo* in budding yeast (Siegers *et al.*, 1999).

Here we establish the co-occurrence of group II chaperonin systems in eukaryotes and archaea by demonstrating that the archaeum *Methanobacterium thermoautotrophicum* contains a homologue of eukaryotic GimC/PFD, which we term MtGimC. We provide evidence that all eukaryotic and archaeal Gim proteins are related coiled coil proteins, and can be grouped into two separate (α and β) classes. Unlike its eukaryotic counterpart, MtGimC contains only one representative subunit from each class. Nevertheless, structural, biochemical and yeast complementation studies reveal that MtGimC and GimC/prefoldin are likely to have a very similar hexameric architecture, for which we present a model. Importantly, MtGimC has the general properties of a molecular chaperone, and similarly to the Hsp70 system, it is able to prevent or retard the aggregation of a number of non-native proteins and to stabilize them for subsequent chaperonin-assisted folding.

Results

Gim proteins are related coiled coil proteins that group into α and β classes

The eukaryotic yeast GimC and bovine PFD complexes each consist of six distinct protein subunits termed Gim1–Gim6 and PFD1–PFD6, respectively (Geissler *et al.*, 1998; Vainberg *et al.*, 1998; Siegers *et al.*, 1999). Closely related

homologues of all six proteins are also found in the nematode *Caenorhabditis elegans*, suggesting that they are likely to be conserved in eukaryotes. Sequence database searches further revealed that Gim1 (PFD6) and Gim5 (PFD5) homologues occur in *Methanococcus jannaschii* (Geissler *et al.*, 1998; Vainberg *et al.*, 1998) as well as in nearly all archaeal genomes sequenced thus far. The only exception appears to be the recently completed genome of *Aeropyrum pernix* (Kawarabayasi *et al.*, 1999), which contains only one Gim subunit, related to Gim1. We term the Gim5 and Gim1 homologues of archaea Gim α and Gim β , respectively. A Gim5 (Gim α) homologue also exists in the evolutionarily deep-branching hyperthermophilic eubacterium *Aquifex aeolicus*, although none of 16 other sequenced eubacterial genomes, including that of the thermophile *Thermotoga maritima*, encode Gim-like proteins.

Although a sequence alignment of Gim/PFD subunits has been presented (Vainberg *et al.*, 1998), a more detailed analysis of the protein sequences as well as secondary structure predictions revealed significant additional insight, namely that all the sequences of Gim proteins can be aligned and form two separate classes. Structure prediction algorithms strongly suggest that Gim proteins encompass three distinct regions: a central region containing β -strands and two extended flanking regions of high helical structure content (Figure 1A and B). The α -helical regions (each ~45 residues in length) are predicted to have a high propensity of forming coiled coils and the core hydrophobic residues of the interacting helices (denoted by the 'a' and 'd' positions in the heptad repeats; reviewed in Lupas, 1996a) can be aligned in all Gim proteins. Importantly, the predicted coiled coil registers coincided exactly with the optimum-score alignment obtained in the multiple sequence alignment program MACAW (Schuler *et al.*, 1991) (Figure 1B).

One notable difference between members of the Gim family is that the Gim2, Gim5 and Gim α proteins in the alignment contain conserved ~25 amino acid insertions not present in the Gim1, 3, 4 and 6 and Gim β proteins (Figure 1A and B). These insertions appear to have arisen from the duplication of a highly conserved region present in all of the Gim subunits that immediately follows the insertion and is predicted to contain two β -strands (the duplicated region is delineated in Figure 1B). This view is supported by the high statistical significance of an alignment between the two regions, whose Poisson probability of chance occurrence is $<10^{-30}$. A comprehensive phylogenetic analysis of Gim proteins from eukaryotes, archaea and *A. aeolicus* revealed the existence of two separate classes of proteins, represented by Gim2/5/ α (which we denote as the α class) and Gim1/3/4/6/ β (β class) (Figure 1C). Most archaea possess only one representative from each class (i.e. Gim α and Gim β , or Gim5 and Gim1 homologues, respectively). The unique eubacterial Gim protein groups with the archaeal Gim α proteins, suggesting that a lateral gene transfer from an archaeum (Nelson *et al.*, 1999) represents the source of the *Aquifex* protein. Interestingly, *M. jannaschii* contains a second Gim α subunit (MjGim α 2), which may also have been acquired by horizontal transfer, and is the nearest phylogenetic neighbour of the *Aquifex* sequence (Figure 1C).

Fig. 1. Gim proteins are related and form separate α and β classes. (A) Schematic representation of Gim subunits based on the sequence alignment and structure predictions in (B). Dashed lines show variable regions, bars denote coiled coils and arrows delineate the positions of β -strands. The β -strand region conserved in all Gim proteins is duplicated in Gim2, Gim5 and Gim α . (B) Sequence alignment of eukaryotic, archaeal and eubacterial Gim proteins. The predicted secondary structure and register of hydrophobic residues in the coiled coil core are shown above the alignment (h = α -helix, s = β -strand; a, d = hydrophobic positions). Hydrophobic residues corresponding to the coiled coil heptad repeat and to the β -strand cores are shown in bold. The position of the duplication is shown by double-headed arrows. DDBJ/EMBL/GenBank accession Nos are indicated next to the sequence names. The coding region of *C.elegans* Gim4 was obtained from the Sanger Centre and was found to contain an insertion of 18 residues not present in other Gim4 sequences. This insertion probably resulted from an incorrect prediction of exon-intron junctions in the gene, since EST sequences of *C.elegans* and the related nematode *Necator americanus*, deposited at NCBI, do not show this insert. It was therefore omitted from the alignment. Archaeal proteins: Mt, *Methanotrophium*; Af, *Archaeoglobus fulgidus*; Ap, *A. permix*; Mj, *M. jannaschii*; Ph, *Pyrococcus horikoshii*. Eukaryotic proteins: Hs, *Homo sapiens*, Sc, *Saccharomyces cerevisiae* and Ce, *C.elegans*. Eubacterial protein: Aa, *A.aerophilus*. (C) Distance-based phylogenetic analysis of Gim proteins. Similar phylogenetic relationships are seen in a maximum likelihood analysis. Significant bootstrap support (>50%) is obtained for individual subunit clusters. Furthermore, homologues of Gim1, 3, 4 and 6 group into the α class and Gim2 and Gim5 group into the β class. While this grouping does not obtain >50% bootstrap support, it is the most likely solution by both methods, with and without inclusion of gapped positions. This finding suggests that the tree is robust and that its shape is not dependent on the information contained in the inserted region. The scale bar represents a distance of PAM10 (0.1).

Fig. 2. Identification of Gim α and Gim β in a high molecular weight complex in *M. thermoautotrophicum*. (A) SDS-PAGE (16%) and Coomassie Blue staining of purified MtGim α and MtGim β proteins (15 μ g each). (B) Western blot of an *M. thermoautotrophicum* crude cell lysate (10 μ g) probed with antibodies raised against MtGim α (α -MtGim α) or MtGim β (α -MtGim β). (C) Fractionation of a *M. thermoautotrophicum* lysate on a Superdex 200 sizing column followed by Western blotting with the antibodies against MtGim α or MtGim β . The majority of the MtGim α and MtGim β proteins co-elute at an estimated molecular weight of ~190 kDa. The positions of molecular weight standards are indicated.

The *M. thermoautotrophicum* Gim α and Gim β subunits are present in a high molecular weight complex *in vivo*

Based on the recent finding that eukaryotic GimC is a hetero-oligomeric molecular chaperone that cooperates with the eukaryotic group II chaperonin TRiC, we sought to establish whether the archaeal *GIM* genes encode components of a structurally and functionally related protein complex. The coding regions of the two *GIM* genes from the archaeum *M. thermoautotrophicum* (Mt) were cloned into bacterial expression vectors, and the two proteins, referred to as MtGim α (Gim5 homologue) and MtGim β (Gim1 homologue), were purified to near homogeneity from *E. coli* overexpressing strains (Figure 2A). Polyclonal antibodies raised against the purified proteins cross-reacted specifically with MtGim α and MtGim β in a crude *M. thermoautotrophicum* lysate (Figure 2B). Quantitative immunoblot analysis showed the amount of MtGim β to be approximately twice that of MtGim α . Together the proteins are quite abundant, making up ~0.4% of the total soluble archaeal proteins. Neither MtGim subunit was found to increase in abundance following 1 h temperature up-shifts of 5 to 15°C from the normal growth temperature of 65°C (data not shown).

Although MtGim α and MtGim β have molecular weights of 15.7 and 13.9 kDa, respectively, analytical size exclusion chromatography (SEC) of an *M. thermoautotrophicum* extract followed by Western blot analysis revealed that the two proteins co-elute at an estimated size of ~190 kDa

(Figure 2C). This result provided the first indication that the two archaeal proteins may form a complex *in vivo*. As judged by SEC, the elution properties of the archaeal Gim proteins is comparable to that of the protein subunits present in the yeast (170–230 kDa) and bovine testis (200 kDa) GimC/PFD complexes (Geissler *et al.*, 1998; Vainberg *et al.*, 1998).

MtGim α and MtGim β assemble into a hexameric complex rich in α -helices

To ascertain whether MtGim α and MtGim β can assemble into hetero-oligomeric complexes similar to eukaryotic GimC/PFD, the two purified, urea-denatured proteins were renatured by dialysis into buffer either individually or together in different ratios, and fractionated by SEC. Aliquots of the fractions were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 3A). MtGim α eluted from the sizing column significantly earlier than MtGim β (estimated molecular weights of 60 and 35 kDa, respectively), suggesting that it assembled into a higher-order structure (Figure 3A, first and second panels). Indeed, cross-linking reactions using bis(sulfosuccinimidyl)suberate (BS³) (Figure 3B) revealed that MtGim α is quantitatively cross-linked as a dimer whereas MtGim β is likely to be monomeric; cross-linking with glutaraldehyde or dimethyl suberimidate (DMS) gave identical results (data not shown). When both unfolded proteins were dialysed together against buffer at a 1:1 molar ratio, the SEC elution profile showed two peaks, one corresponding to MtGim α dimer (Figure 3A, third panel, fractions 18 and 19) and another to a higher molecular weight species of ~190 kDa containing both proteins (fractions 15 and 16). Notably, the latter protein complex eluted at the same position on the Superdex 200 column as was observed for the MtGim α and MtGim β proteins present in the archaeal cell lysate (compare Figure 2C with Figure 3A, third panel). Combining MtGim β and MtGim α in a 2:1 ratio gave rise only to the high molecular weight complex (Figure 3A, fourth panel). At a higher ratio of MtGim β over MtGim α (3:1), uncomplexed MtGim β was observed in addition to the complex (Figure 3A, fifth panel). From these studies, we conclude that the complex contains MtGim β and MtGim α in a 2:1 ratio, a finding that is in agreement with the quantitative amino acid analysis of the complex (data not shown) and the relative intensities of the two subunits in the complex upon staining with Coomassie Brilliant Blue. Assembly of the complex was equally efficient when refolded MtGim α was added to refolded MtGim β , providing evidence that the MtGim α dimer serves as a nucleus onto which MtGim β monomers assemble. We refer to the ~190 kDa species formed *in vitro* and present *in vivo* as the MtGim Complex, or MtGimC.

The subunit composition of the complex could be resolved by sedimentation equilibrium measurements, which allow the native molecular mass of a protein species to be determined independently of its shape. Three ultracentrifugation runs at different protein concentrations were performed. The data acquired (Figure 3C) fit well to a monodisperse model and showed that purified MtGimC contains only one species possessing a mass of ~83.7 kDa. Given the observed 2:1 ratio for the β and α subunits in the complex, this value is, within experimental error,

Fig. 3. Assembly of MtGim α and MtGim β into a hexameric complex with high α -helical content. **(A)** Size exclusion chromatography (on a Superdex 200 column) of refolded MtGim β , MtGim α , and mixtures of MtGim β and MtGim α in 1:1, 2:1 and 3:1 molar ratios. The left panel shows fractions analysed by SDS-PAGE (16%) and Coomassie Blue staining, and the right panel shows the elution profiles (A_{280}) of the samples containing different MtGim β to MtGim α ratios. MtGimC represents the complex between MtGim α and MtGim β . **(B)** Cross-linking reactions of MtGim α and MtGim β (each 20 μ M) using 1 mM BS³ for 1 h, analysed by SDS-PAGE and Coomassie Blue staining. **(C)** Sedimentation equilibrium analysis of purified MtGimC performed at three different protein concentrations (see Materials and methods). R_M was 5.9, 6.4 and 6.9 cm for the high, intermediate and low concentrations, respectively. The data obtained fit well to a single ideal species with a molecular mass of 83.7 kDa. **(D)** Circular dichroism measurements of MtGim α , MtGim β and MtGimC in 20 mM NaPO₄ pH 8.0 at 20°C. All of the samples examined have a high α -helical structure content; calculations suggest that MtGimC has an α -helical content of $\sim$ 85%.

consistent with a hexameric particle composed of four molecules of MtGim β and two molecules of MtGim α , and having a total calculated molecular weight of 86.8 kDa. The overestimated size of MtGimC by SEC ($\sim$ 190 kDa)

compared with the ultracentrifugation results suggests that the complex has a non-globular structure. Since the migration of the six-subunit yeast and bovine GimC/PFD complexes during SEC parallels that of MtGimC, we

propose that the eukaryotic assemblies are hexameric as well.

To provide evidence for the presence of the extensive α -helical coiled coil regions that are predicted in both archaeal Gim proteins (see Figure 1A and B), we performed far-UV circular dichroism (CD) on the individual MtGim proteins as well as on the complex. All spectra obtained were highly characteristic of proteins containing mostly α -helices, possessing pronounced minima at 208 and 222 nm and a maximum at ~ 192 nm (Figure 3D). The spectrum of MtGimC approximates the spectra of both subunits in isolation, suggesting that only marginal conformational changes occur to the subunits upon incorporation into the complex. Overall, the CD spectra and predicted structures of MtGim α and MtGim β are very similar, and we also note that the presence of extensive, possibly uninterrupted coiled coil structures in MtGimC would be consistent with extended shapes for the individual subunits and the complex.

Functional conservation of the archaeal and eukaryotic Gim complexes

The presence of two homologous subunits (Gim1 and Gim5) within a common hexameric structure suggests that eukaryotic and archaeal Gim complexes are closely related. In order to characterize the structural and functional relationships between the two, we tested whether MtGim α and MtGim β were capable of complementing yeast strains lacking the homologous subunits Gim5 and Gim1, respectively. We first observed that the two archaeal proteins can be produced in yeast (see Figure 4B) and assemble into a complex when co-produced, as established by co-immunoprecipitation experiments with affinity-purified antibodies that cross-react specifically against MtGim α and MtGim β (data not shown).

As documented in Figure 4A and as reported previously (Geissler *et al.*, 1998), yeast $\Delta gim1$ and $\Delta gim5$ strains are sensitive to the microtubule-depolymerizing drug benomyl. This phenotype in the $\Delta gim1$ yeast can be fully complemented by transforming the strain with plasmids expressing yeast or mouse *GIM1*. MtGIM β (the *GIM1* homologue) expressed in this strain was also able to partially rescue the benomyl sensitivity (Figure 4A, top panels). This effect was specific, as expression of MtGIM α (the *GIM5* homologue) in the $\Delta gim1$ strain produced no visible changes in growth behaviour, and co-expression of both archaeal genes in the same strain was no more effective than expression of MtGIM β alone. Remarkably, MtGIM β was also able to partially complement a yeast mutant lacking *GIM4*, another member of the β class of Gim subunits (data are only shown for *GIM1*), despite the low overall sequence homology between the two proteins (see Figure 1B). The positive effect of MtGIM β expression in the yeast $\Delta gim1$ strain could be directly attributed to the incorporation of MtGim β into the Gim1-deficient yeast Gim complex, as shown by co-immunoprecipitation experiments with antibodies against yeast Gim2, an α class subunit (Figure 4B, top right panel). In contrast, expression of MtGim α had no detectable rescuing effect in the $\Delta gim5$ strain (Figure 4A, bottom panels), even though the archaeal protein could be co-immunoprecipitated with yeast Gim2 in the Gim5-deficient yeast cells (Figure 4B, top left panel). Parallel experiments performed

Fig. 4. Functional conservation of the yeast and archaeal Gim complexes. (A) Complementation analyses of *S. cerevisiae* $\Delta gim1$ and $\Delta gim5$ strains using MtGIM β and MtGIM α . Both $\Delta gim1$ and $\Delta gim5$ yeast strains are highly sensitive to a 2.5 μ g/ml concentration of the microtubule depolymerizing drug benomyl (a in top panel and g in lower panel, respectively). This phenotype is rescued entirely by expression of yeast (Sc) and murine (Mm) *GIM1* (b and c), and partially complemented by expression of archaeal MtGIM β (d). The partial complementation by MtGim β is specific, as MtGim α alone has no effect on benomyl sensitivity (e), and co-expression of MtGIM α with MtGIM β does not enhance the complementation by MtGIM β (f). While ScGim5 rescues benomyl sensitivity in the $\Delta gim5$ strain (h), neither MtGIM α (i) nor MtGIM β (j) nor a combination of both (k) complement the null mutant. (B) MtGIM α and MtGIM β expressed in yeast $\Delta gim5$ and $\Delta gim1$ strains, respectively, can be co-immunoprecipitated with an antibody against yeast Gim2 (left and right panels, respectively). MtGim α or MtGim β proteins were expressed in strains lacking yeast *GIM5* and *GIM1*, respectively, and co-immunoprecipitated with antibodies against yeast ScGim2, followed by Western blotting with anti-MtGim α or anti-MtGim β antibodies (upper panels). Control immunoprecipitation reactions performed in cells further deleted of *GIM2* (bottom panels) show that MtGim α and MtGim β co-immunoprecipitate specifically with ScGim2 [lane 1, cytosolic extract; lane 2, supernatant (sup) after the immunoprecipitation (IP); lane 3, immune pellet].

in $\Delta gim2$ yeast strains expressing either MtGim α or MtGim β (and lacking yeast *GIM5* and *GIM1*, respectively) showed that the archaeal proteins were immunoprecipitated specifically with yeast Gim2, i.e. only when the yeast Gim2 protein was present (Figure 4B, bottom two panels). Together, these data confirm that MtGim α and MtGim β are structural homologues of the yeast Gim5 and Gim1/Gim4 proteins, respectively, and that the function of MtGim β is similar to that of the yeast subunits Gim1 and Gim4. The reason for the inability of MtGim α to partially complement the $\Delta gim5$ strain may be due to the fact that although Gim α can associate with Gim2 (of the α class), other yeast subunits of the β class may not assemble properly onto Gim α due to possible structural differences in the yeast and archaeal subunits.

We also observed that co-expressing both archaeal *GIM* genes had no effect on the benomyl sensitivity of yeast entirely lacking endogenous GimC (K.Siegers and E.Schiebel, manuscript in preparation). Since the archaeal protein complex appears to be functional at the growth temperature of yeast (see below), our data suggest that eukaryotic GimC/PFD may have evolved specialized functions that cannot be performed by its archaeal counterpart.

MtGimC interacts with a range of non-native proteins

Yeast strains lacking individual or multiple Gim subunits show specific defects in microtubule and actin biogenesis (Geissler *et al.*, 1998; Vainberg *et al.*, 1998; Siegers *et al.*, 1999). Several non-cytoskeletal proteins translated in reticulocyte lysate do not appear to interact with either TRiC or its cofactor GimC/PFD (Hansen *et al.*, 1999). These data suggest that GimC/PFD interacts selectively with actin and tubulins, and may not bind to non-native proteins in a general manner as do many molecular chaperones. We therefore investigated whether MtGimC has the properties of a general chaperone by using a number of different substrates and experimental conditions.

We first observed that MtGimC forms a complex with unfolded actin, a property comparable to that of eukaryotic GimC/PFD (Vainberg *et al.*, 1998). Dilution of ^{35}S -labelled, urea-denatured mouse β -actin (D-*actin) into a buffer solution containing MtGimC gave rise to a distinct complex that can be detected on a 4.5% non-denaturing gel (Figure 5A, lane 1). In contrast, D-*actin diluted into buffer alone forms aggregates that are not resolved on the native gel (Figure 5A, lane 2; Melki and Cowan, 1994). Similarly, only a smear of aggregated material could be detected on the native gel if the denatured actin was added to the individual MtGim α or MtGim β subunits (Figure 5A, lanes 3 and 4). The position of migration of GimC ('C') on the native gel coincided with the position of the labelled actin, whereas the positions corresponding to the individual Gim α (' α ') and Gim β (' β ') subunits showed no labelled actin (Figure 5A, arrowheads on the right of the panel). The fact that not all of the denatured actin is recovered as a complex with MtGimC (hence the presence of unbound actin in the native gel) suggests that its affinity for substrates is lower relative to GroEL (see also Figure 6).

Next, we tested the ability of MtGimC to prevent the aggregation of three unfolded proteins, hen lysozyme (14 kDa), bovine mitochondrial rhodanese (33 kDa) as well

as glucose dehydrogenase (39 kDa) from the archaeum *Thermoplasma acidophilum*. Reduced guanidine-denatured lysozyme aggregates when diluted 100-fold into a buffer solution, as measured by the increase in light scattering detected at 320 nm over a period of 10 min at 25°C (Figure 5B, $\diamond$). MtGimC efficiently suppressed the aggregation of lysozyme in a concentration-dependent manner until little or no aggregation could be detected when the archaeal protein complex was present at an equimolar (or greater) concentration relative to the denatured protein (Figure 5B, filled symbols). MtGimC also prevented the aggregation of chemically unfolded rhodanese with similar efficiency (Figure 5C). The assembled MtGim complex was found to be specifically required for chaperone function, as neither the individual MtGim proteins nor bovine serum albumin (BSA) or IgG prevented lysozyme or rhodanese aggregation at concentrations where MtGimC completely inhibited aggregation (data not shown). Importantly, this function of MtGimC could also be demonstrated for the thermophilic protein glucose dehydrogenase (Figure 5D). Furthermore, complexes of MtGimC with non-native human dihydrofolate reductase (DHFR, 23 kDa) and firefly luciferase (62 kDa), but not with the corresponding native proteins, were also observed by native PAGE (data not shown).

MtGimC was also efficient in preventing the thermally induced aggregation of rhodanese at 42°C (Figure 5C, inset) and a complex between MtGimC and the denatured protein could be isolated by gel filtration (Figure 5E). The rhodanese bound to MtGimC was in an unfolded conformation as it was much more sensitive to proteinase K treatment than native rhodanese (under conditions where MtGimC was resistant to the protease and functional; data not shown) (Figure 5E). Overall, the above findings strongly suggest that MtGimC is a bona fide molecular chaperone that has the ability to interact with and stabilize a wide variety of proteins in a non-native but folding-competent conformation (see below).

MtGimC stabilizes non-native proteins for subsequent chaperonin-assisted folding

Eukaryotic GimC/PFD can bind unfolded actin *in vitro* and transfer it to TRiC for refolding to the native state (Vainberg *et al.*, 1998). This function has been attributed to a specific interaction between GimC/PFD and TRiC (Vainberg *et al.*, 1998; Siegers *et al.*, 1999). Somewhat unexpectedly, we were unable to detect a direct physical interaction between MtGimC and archaeal chaperonins from different sources under a number of experimental conditions, including incubation of the proteins at high temperatures. Although we cannot exclude a transient interaction between archaeal GimC and thermosome, it is possible that this specific function has been acquired only later in evolution. Nevertheless, we found that substrates bound to MtGimC can be released to and folded by two different chaperonin systems.

A transfer of MtGimC-bound actin to group I and group II chaperonins was observed upon incubation of the MtGimC-actin complex with GroEL or the thermosome from *T. acidophilum*, respectively (Figure 6A), but neither GroEL (with or without GroES) (Tian *et al.*, 1995) nor the archaeal chaperonin was able to refold some of this actin in the presence of ATP at 37°C (data not

Fig. 5. MtGimC interacts with and stabilizes a broad range of non-native proteins. **(A)** MtGimC forms a complex with chemically unfolded actin. Urea-denatured, 35 S-labelled murine β -actin (D-actin) was diluted 100-fold into buffer containing $\sim 3 \mu$ M MtGimC (lane 1), no protein (lane 2), $\sim 10 \mu$ M MtGim α (lane 3) or $\sim 10 \mu$ M MtGim β (lane 4) and incubated at 30°C for 15 min. Samples were then separated by 4.5% native PAGE and autoradiographed. Note that in the case where the denatured actin is added to buffer or to the individual MtGim α or MtGim β proteins, only actin aggregates are visible as a smear. The positions where the MtGim α (' α '), MtGim β (' β ') and the complex ('C') migrate on the native gel (as seen by Coomassie Blue staining) is shown by the arrowheads on the right of the panel. **(B)** MtGimC can suppress the aggregation of reduced, urea-denatured lysozyme (D-lysozyme) in a concentration-dependent manner. A 160 μ M solution of D-lysozyme was diluted 100-fold into buffer without (open symbol) or with an increasing concentration of MtGimC as indicated (closed symbols), and aggregation of lysozyme was monitored by measuring light scattering at 320 nm over a period of 10 min. **(C)** MtGimC prevents the aggregation of a 1 μ M solution of chemically denatured rhodanese (D-rhodanese), as described above for lysozyme. At 42°C , a 1 μ M solution of native rhodanese is also prevented from aggregating when incubated together with 1.25 μ M MtGimC (inset). **(D)** The aggregation of guanidine-denatured *T. acidophilum* (archaeal) glucose dehydrogenase (GD) is significantly decreased when diluted into buffer containing an equimolar amount of MtGimC. The bar graph shows the relative amounts of GD aggregated after 10 min as measured by light scattering. GD (2 μ M final concentration) was diluted into buffer alone (lane 1), or into a solution containing 4 μ M of the control protein IgG (lane 2) or 2 μ M MtGimC (lane 3). **(E)** Rhodanese in a complex with MtGimC is sensitive to proteinase K. The upper three panels show the position of migration of native rhodanese (N-rho), denatured rhodanese (D-rho), and the MtGimC–rhodanese complex on a Superdex 200 gel filtration column, as detected by Western blotting with an anti-rhodanese antibody. The chemically-denatured rhodanese aggregates upon dilution into buffer and is not recovered on the sizing column after a centrifugation step; when incubated together, MtGimC and D-rhodanese form a complex that migrates as a distinct species on the column. This MtGimC-bound rhodanese (fraction 7) is in a non-native conformation based on its sensitivity to a 10 μ g/ml proteinase K digestion on ice for 10 min (lower left panel), relative to that of native rhodanese (fraction 10) treated identically (lower right panel).

shown). Since natural substrates of the thermosome are as yet unknown, we focused our analysis on the cooperation between MtGimC and the closely thermosome-related eukaryotic group II chaperonin TRiC, using actin as a model substrate. Although the binding of denatured actin and release of folded actin by TRiC is not as efficient *in vitro* as *in vivo* (Siegers *et al.*, 1999), the production of native actin is strictly TRiC-dependent and can be detected by separation of the reaction products on non-denaturing gels (e.g. a typical actin refolding reaction with TRiC and ATP is shown in Figure 6B, lane 3; see also Melki and Cowan, 1994).

We observed that non-native actin was stabilized by MtGimC (but not the control protein IgG) for at least 15 min and could subsequently be transferred to and

refolded by TRiC in the presence of ATP (Figure 6B, lanes 1, 2 and 6). The actin produced formed a complex with DNase I (Figure 6B, lane 7), demonstrating that it was in a monomeric, folded conformation (Lazarides and Lindberg, 1974; Kabsch *et al.*, 1990). In contrast to MtGimC, the control protein IgG was not able to maintain non-native actin in a form competent for folding by TRiC, even though some of the denatured actin present in the mixture with IgG was bound by TRiC (Figure 6B, lane 2). Order-of-addition experiments showed further that actin refolding by TRiC occurred with similar efficiency in the following three cases: (i) when unfolded actin was added to TRiC alone (Figure 6B, lane 3); (ii) when MtGimC was added to the pre-formed TRiC–actin complex (Figure 6B, lane 4); and (iii) when unfolded actin was

Fig. 6. MtGimC captures non-native proteins and releases them to chaperonins for folding to the native state. (A) MtGimC-bound D-actin can be released to group I (GroEL) and group II (archaeal thermosome) chaperonins. MtGimC-labelled actin complexes were prepared as described in Figure 5. These were then incubated with 3 µM GroEL (left panel) or 3 µM thermosome (middle panel) for 15 min at 30°C and analysed by 4.5% native PAGE run in 80 mM MOPS–KOH pH 7.0. Note in each case the disappearance of the labelled actin from MtGimC (left and middle panels). The thermosome does not enter the MOPS native gel but does form a complex with the denatured actin, as detected on a Tris-based native gel (right panel). (B) D-actin was added to a solution containing 6 µM IgG (lanes 1 and 2), 0.3 µM TRiC (lanes 3–5) and/or 2 µM MtGimC (lanes 5, 6 and 7) and incubated for 15 min at 30°C. All reactions were supplemented with 1 mM Mg-ATP and to some, TRiC (lanes 2, 6 and 7) or MtGimC (lane 4) was added. Folding reactions were carried out for 60 min at 30°C and analysed by native PAGE and autoradiography. The position of migration of native actin (N-actin) on the native gel is indicated. DNase I, which forms a complex with native but not non-native actin, was added to the reaction in lane 7 prior to native PAGE (notice the shift in the native actin). (C) MtGimC prevents rhodanese from aggregating and transfers it to GroEL. Chemically unfolded rhodanese (100 µM; D-rhodanese) was diluted 100-fold into a solution containing MtGimC (2 µM) and incubated for 15 min at 25°C. Subsequently, GroEL (1 µM) was added and the reaction analysed by SEC (left panel; rhodanese co-migrates with GroEL in lanes 5 and 6). Alternatively, D-rhodanese was incubated with buffer alone (or with buffer containing a control protein; not shown) for 15 min as above, followed by the addition of GroEL (right panel; little or no rhodanese is bound to GroEL). (D) The rhodanese transferred from MtGimC to GroEL is competent to release the native state. Chemically denatured rhodanese (100 µM) was diluted 100-fold into solutions containing buffer only (□ and ○), 2 µM MtGimC (▼ and ■) or 0.5 µM GroEL (△ and ◆) and incubated for 15 min at 25°C. After removing rhodanese aggregates by centrifugation, MtGimC was added to reactions already containing GroEL (◆), and GroEL was added to some of the reactions previously containing MtGimC (■) and buffer (○). Folding reactions were initiated by the addition of GroES (to 1 µM) and/or ATP (to 5 mM). At the time points indicated, aliquots were assayed for rhodanese activity. Results are plotted as a percentage of the activity of a 1 µM native rhodanese control.

added to a combination of TRiC and MtGimC (Figure 6B, lane 5). In all cases, folding was then allowed to proceed with the addition of ATP for 1 h at 30°C.

Building on our finding that MtGimC prevents the aggregation of unfolded rhodanese and forms a complex with the denatured protein (Figure 5C and E), we tested whether MtGimC can also act in a sequential folding pathway with GroEL/GroES. The refolding of rhodanese from denaturant is known to depend on both GroEL and GroES, and on ATP (Martin *et al.*, 1991). The ability to maintain unfolded rhodanese competent for subsequent folding by GroEL/GroES has been demonstrated previously for the eubacterial Hsp70 system consisting of DnaK, DnaJ and GrpE (Langer *et al.*, 1992).

When added to a solution containing MtGimC, unfolded rhodanese was prevented from aggregating (as reported

in Figure 5C) and was quantitatively transferred to GroEL when the latter chaperone was added (i.e. the rhodanese co-migrated only with GroEL during SEC; Figure 6C, left panel). On the other hand, when unfolded rhodanese was added to buffer alone, essentially all of the rhodanese aggregated and was not competent to bind GroEL (Figure 6C, right panel). The amount of rhodanese that bound to GroEL upon transfer from MtGimC was essentially the same as when the unfolded protein was added directly to GroEL (data not shown). Accordingly, the efficiency of rhodanese refolding by GroEL/GroES/ATP was similar in both cases (Figure 6D, ■ and △, respectively), indicating that the rhodanese transferred from MtGimC to GroEL had been maintained in a folding-competent state. MtGimC had little effect on the folding of rhodanese by GroEL when added after the rhodanese

was already bound to the chaperonin (Figure 6D, ◆). Control reactions showed that when rhodanese was diluted from denaturant into buffer alone or into a solution containing IgG, aggregates formed that could not be captured by GroEL; further addition of GroES and ATP to this material did not yield any more folded rhodanese than the ~10% that refolds spontaneously (Figure 6D, ○ and □).

As with denatured actin as the substrate, no detectable amount of denatured rhodanese was folded by MtGimC alone in the presence or absence of ATP (Figure 6D, ▼). In contrast to GroEL (data not shown), MtGimC did not prevent the spontaneous refolding of rhodanese that occurs upon dilution from denaturant, suggesting that MtGimC has a lower overall affinity for the refolding protein than GroEL (Figure 6D, ▼). In addition, MtGimC did not enhance the ATP-dependent refolding of rhodanese by GroEL in the absence of GroES (data not shown). Taken together, our functional data show that MtGimC exerts its chaperone effect on actin and rhodanese folding by first capturing and stabilizing aggregation-sensitive folding intermediates, and then making them available to group I and II chaperonins, respectively, for folding to the native state.

Discussion

Chaperonins have acquired different functional mechanisms in the lineages leading to present day eubacteria and archaea/eukarya: group I chaperonins (e.g. GroEL from *E.coli*) assist in protein folding in cooperation with a GroES-type cofactor, whereas group II chaperonins (eukaryotic TRiC and archaeal thermosome) function independently of a GroES-like protein. The recent discovery of a eukaryotic protein complex, termed GimC or PFD, which interacts with non-native actin and tubulin, and associates with TRiC during the folding of these proteins (Geissler *et al.*, 1998; Vainberg *et al.*, 1998; Hansen *et al.*, 1999; Siegers *et al.*, 1999), suggested that TRiC utilizes an ancillary cofactor that operates in a manner distinct from GroES. Here, we have shown that the cytosol of the archaeum *M.thermoautotrophicum* contains an abundant hetero-oligomeric molecular chaperone complex, MtGimC, which is related to eukaryotic GimC/PFD, establishing the co-occurrence of GimC and group II chaperonins in archaea and eukarya. Our data support the notion that the overall structure of archaeal and eukaryotic GimC is a conserved hexamer built from two separate classes of Gim subunits. While eukaryotic GimC may interact with a limited number of target proteins, MtGimC binds a wide range of non-native proteins and stabilizes them for subsequent interaction with the chaperonin, suggesting that the archaeal GimC chaperone has the potential to play a general role in *de novo* protein folding. *In vivo* experiments will be necessary to explore this possibility further.

Structural model for GimC/prefoldin

MtGimC is a hexamer composed of two molecules of MtGim α and four molecules of MtGim β (Figure 3). The N- and C-terminal regions of the two subunits, as well as of the eukaryotic Gim proteins, are predicted to be α -helical in structure and to form coiled coils, consistent

Fig. 7. Structural model for archaeal and eukaryotic Gim complexes. The hexameric Gim complexes from both archaea and eukaryotes are built from two classes of subunits (α and β). In archaea, the α class comprises Gim α , whose closest eukaryotic homologue is Gim5. The β class is represented by Gim β , whose closest eukaryotic homologue is Gim1. In eukaryotes, the α class includes Gim2 and Gim5, while the β class contains the Gim1, Gim3, Gim4 and Gim6 subunits. The grouping into two separate classes is based on sequence comparisons and a phylogenetic analysis, as presented in Figure 1. Gim α from the archaeum *M.thermoautotrophicum* exists as a dimer in isolation, and Gim β as a monomer. When mixed, four β class subunits assemble onto the 'core' α class dimer to form a hexamer. Based on their shared primary and predicted secondary structures, as well as complementation studies (Figure 4), the archaeal and yeast α and β class subunits are likely to be conserved. Thus, the eukaryotic subunits Gim1, Gim3, Gim4 and Gim6 would be expected to assemble onto a core of Gim2/Gim5 to form the hexameric complex.

with circular dichroism measurements (Figures 1 and 3). Our reconstitution and cross-linking experiments revealed that the two archaeal α subunits associate with each other to form a 'platform' onto which four β subunits assemble. Given that the β subunits do not detectably self-associate, we assume that in the final complex they make contacts mainly to the α subunits, resulting in the topological model for MtGimC shown in Figure 7. Although we cannot exclude the possibility that one Gim β subunit may, through contact with an α subunit, undergo structural rearrangements that may induce dimerization with another β subunit (leading to a linear arrangement of subunits, i.e. $\beta\beta\alpha\alpha\beta\beta$), we regard this as unlikely. The general architecture of archaeal and eukaryotic GimC/PFD is likely to be conserved, based on the finding that the archaeal α and β subunits are the representative members of two classes of Gim subunits: the α class comprises subunits 2 and 5, and the β class subunits 1, 3, 4 and 6 of GimC/PFD (Figure 1). Accordingly, the eukaryotic complex would consist of a dimer of Gim2 and Gim5, forming a core around which the Gim1, 3, 4 and 6 subunits associate (Figure 7). That the archaeal and eukaryotic Gim complexes are structurally related is supported by the finding that both MtGim α and MtGim β can be co-immunoprecipitated with yeast Gim2 when expressed in strains lacking *GIM5* and *GIM1*, respectively, and that MtGim β is able to partially complement defects in two yeast β class subunits, namely Gim1 and Gim4 (Figure 4).

Role of MtGimC in protein folding

Our data indicate that MtGimC has properties of a general chaperone in that it is able to prevent the aggregation and stabilize the non-native states of a variety of proteins (Figures 5 and 6). In contrast, eukaryotic GimC/PFD is thought to be more specific for the main substrates of TRiC, actin and tubulins (Geissler *et al.*, 1998; Vainberg *et al.*, 1998; Hansen *et al.*, 1999; Siegers *et al.*, 1999). Notably, MtGimC alone was insufficient to mediate the re-folding of denatured polypeptides, such as the model protein rhodanese. Given that unfolded rhodanese was readily transferred from MtGimC to GroEL, which has a higher affinity for the substrate, it seems plausible that MtGimC has rapid on and off rates for non-native protein, allowing it to function by a partitioning mechanism that is independent of nucleotide-dependent regulation. Slow folding, aggregation-sensitive proteins that are chaperonin-dependent, such as rhodanese, would rebind to MtGimC before they have been able to reach their native state (or before they aggregate) in free solution. In contrast, rapidly folding polypeptides, which are chaperonin independent, would probably interact only transiently with MtGimC.

The proposed mechanism of substrate transfer from MtGimC to chaperonin is independent of a direct interaction between the two components. Although a specific association of archaeal GimC with the eubacterial group I chaperonin GroEL is not expected, MtGimC and GroEL are able to cooperate in rhodanese folding *in vitro*. In contrast, eukaryotic GimC/PFD has been suggested to form a complex with TRiC, but not with GroEL, resulting in specific substrate transfer to TRiC (Vainberg *et al.*, 1998; Siegers *et al.*, 1999). In any case, GimC/PFD binds to TRiC (Vainberg *et al.*, 1998; Siegers *et al.*, 1999), resulting in an intimate cooperation with TRiC during folding (Siegers *et al.*, 1999). Such an interaction with the group II chaperonin TRiC was not found for MtGimC in the present study, suggesting that the archaeal chaperone may only fulfil part of the function of GimC/PFD. It is possible that the ability of GimC/PFD to interact directly with the chaperonin TRiC is related to the increase in subunit complexity that occurred during the co-evolution of the eukaryotic proteins. Indeed, although MtGimC assembles properly upon expression in yeast, it is unable to replace functionally the endogenous yeast complex (K.Siegers and E.Schiebel, manuscript in preparation).

De novo protein folding in eubacteria, eukarya and archaea

Recent studies in eubacteria and eukarya have defined some of the essential players in chaperone-assisted protein folding in the cytosol. In *E.coli*, the chaperone and prolyl-*cis/trans*-isomerase trigger factor cooperates with the Hsp70 system (DnaK, DnaJ, GrpE) in stabilizing a large fraction of newly synthesized polypeptides during translation to facilitate efficient folding upon completion of synthesis (Deuerling *et al.*, 1999; Teter *et al.*, 1999). A subset of newly synthesized chains (~10% of total) interact post-translationally with GroEL to fold in the protected cavity of the chaperonin in a GroES-dependent process (Ewalt *et al.*, 1997). Similarly, eukaryotic Hsp70 interacts extensively with nascent polypeptides (Beckmann *et al.*, 1990; Pfund *et al.*, 1998; Thulasiraman *et al.*, 1999) and the chaperonin TRiC is required for the folding of a subset

of proteins, including actin and tubulins (Sternlicht *et al.*, 1993; Hynes *et al.*, 1996; Siegers *et al.*, 1999; Thulasiraman *et al.*, 1999). Although eukaryotes lack a homologue of trigger factor, the ribosome-bound protein complex NAC (Wiedmann *et al.*, 1994) may have an analogous role in protecting nascent chains.

In comparison, little is known about the components responsible for *de novo* protein folding in the cytosol of archaea (Macario and de Macario, 1999). Prior to this study, the only molecular chaperone known to be ubiquitously present in archaea was the chaperonin (thermosome); we have not detected NAC or trigger factor genes in database searches, and remarkably, archaea are aboriginally devoid of an Hsp70 system, although approximately one-third of 20 species examined have apparently acquired one through lateral gene transfers with bacteria (Gribaldo *et al.*, 1999; Macario and de Macario, 1999). In contrast, archaeal Gim proteins are found in all sequenced archaeal genomes (Figure 1), and we have shown that similarly to DnaK/Hsp70, MtGimC can interact with and stabilize a number of non-native proteins (Figure 5). The ability of MtGimC to release folding intermediates to a chaperonin for folding to the native state (Figure 6) is also one of the functions of the DnaK/Hsp70 system in the context of *de novo* protein folding (Langer *et al.*, 1992; Frydman *et al.*, 1994; Teter *et al.*, 1999). Thus, the archaeal Gim complex may be generally involved in protein biogenesis in cooperation with the thermosome, a function that may be shared with Hsp70, if present in the organism, and presumably with other archaeal chaperones that remain to be identified. To test this hypothesis, the flux of newly synthesized polypeptides through the GimC/chaperonin system will have to be determined *in vivo* in archaea containing or lacking Hsp70.

Materials and methods

Cloning, expression and purification of MtGim α and MtGim β

The coding regions for MtGim α and MtGim β (DDBJ/EMBL/GenBank accession Nos gi2622734 and gi2621763, respectively) were amplified by PCR from *M.thermoautotrophicum* Δ H genomic DNA using combinations of oligonucleotides with *Nde*I and *Bam*HI restriction sites: 5'-CACTACATATGGAAGACCAGCAGAGGCTCGAGGAGATAGTG-3' and 5'-CACTAGGATCCTTACTCTCCACTTCCCCTTACCTTTTGAGGAG-3' for MtGim α ; 5'-CACTACATATGGAACCTCCACA-GAATGTACAGCACCAG-3' and 5'-CACTAGGATCCTTAGTTACCATCCCAGGATTGATACCCGC-3' for MtGim β . The PCR products were subcloned into the bacterial expression vector pRSET6a (Schoepfer, 1993) cut with *Nde*I–*Bam*HI. BL21(DE3) cells (Studier *et al.*, 1990) were transformed with the two constructs, grown at 30°C (MtGim α) or at 37°C (MtGim β) in LB medium (with 100 μ g/ml ampicillin) to an OD₆₀₀ of ~0.7, induced with 1 mM IPTG, and harvested after 3–5 h. Cells were resuspended in lysis buffer [50 mM Tris pH 8.0, 10 mM NaCl, 2 mM dithiothreitol (DTT), 1× Boehringer Mannheim complete protease inhibitors] and disrupted by freeze-thawing and sonication. MtGim α , expressed in soluble form, was purified by successive chromatographic steps on Q-Sepharose, phenyl Sepharose, heparin (collected in flowthrough), and S-100HR columns. The latter purification was performed under denaturing conditions in buffer A (8 M urea, 20 mM Tris-HCl pH 8.0). MtGim β was purified from isolated inclusion bodies by resolubilization in buffer A and chromatography on Q-Sepharose and S-100HR (Pharmacia) columns under denaturing conditions. The purity of the proteins was assessed by 16% SDS-PAGE and Coomassie Blue staining. The identities of the proteins were verified by N-terminal sequencing, amino acid analysis and mass spectrometry.

Production of polyclonal antibodies and Western blot analyses

MtGim α and MtGim β (0.5 mg each) were injected into rabbits first with Freund's complete adjuvant and then with incomplete adjuvant (Sigma)

at intervals of 2–3 weeks, and serum was checked for cross-reactivity against *M. thermoautotrophicum* lysate using purified MtGim proteins as standards. Antibodies were affinity-purified on Affi-Gel 15 columns coupled with the appropriate MtGim proteins as described by the manufacturer (Bio-Rad).

Western blot analyses were carried out in TBS-T (25 mM Tris-HCl, 140 mM NaCl, 0.05% Tween 20 pH 7.4) with 1000-fold dilutions of primary polyclonal (anti-MtGim α , anti-MtGim β or anti-rhodanese) and then secondary (HRP-conjugated anti-rabbit) antibodies, followed by immunodetection with Amersham's ECL kit.

Size exclusion chromatography (SEC)

Samples of 50 or 100 μ l were fractionated on a Superdex[®]200 PC3.2/30 column at room temperature using buffers as specified in the appropriate sections (flow rate 40 μ l/min; fraction sizes 50 or 100 μ l). The column was calibrated with the following molecular weight protein standards: catalase (232 kDa), aldolase (158 kDa), BSA (67 kDa), carbonic anhydrase (29 kDa) and α -lactalbumin (14 kDa) (all from Pharmacia). An *M. thermoautotrophicum* crude cell extract was prepared by lysing cells in buffer L (50 mM Tris pH 7.5, 150 mM NaCl, 1 $\times$ complete protease inhibitor) in a French press and pelleting the cellular debris. For SEC, ~300 μ g of the protein lysate were applied.

Assembly of MtGimC

The purified MtGim α and MtGim β proteins were refolded separately at concentrations between 10 and 100 μ M each, or together in different ratios (keeping MtGim β constant at 50 μ M), by dialysis in buffer T (20 mM Tris pH 8.0) at 4°C. Samples of 50 μ l were analysed by SEC using buffer T as described above. Large-scale preparations of the complex were performed by combining MtGim β (100 μ M) at a 2:2 molar ratio over MtGim α and performing equilibrium dialysis in buffer T. MtGimC was concentrated with a Centrprep 30 (Amicon), purified over an S-200HR 26/60 column in buffer T, and stored at –80°C with 30% glycerol. Before use, MtGimC was dialysed against an appropriate buffer.

Cross-linking analysis

MtGim α and MtGim β proteins (20 μ M each) were dialysed against CL buffer (50 mM NaPO₄ pH 7.4, 100 mM NaCl, 1 mM MgCl₂ and 1 mM DTT) and cross-linked by incubation with 1 mM BS³ [bis(sulfosuccinimidyl)suberate; Pierce] for 1 h at room temperature. Reactions were analysed by SDS-PAGE and Coomassie Blue staining.

Sedimentation equilibrium measurements

Sedimentation equilibrium experiments were performed in a Beckman Optima XL-A ultracentrifuge using an An60Ti rotor with a 6 channel cell centerpiece. Three 200 μ l samples of MtGimC containing different protein concentrations (A_{280} of 0.6, 0.3 and 0.15) in 20 mM NaPO₄ pH 8.0/100 mM NaCl were analysed at 9000 r.p.m. and 20°C until the samples reached equilibrium. The radial distribution of MtGimC was monitored by absorbance at 280 nm. Five scans were averaged and analysed using the ORIGIN software (Beckman).

Circular dichroism measurements

CD experiments were carried out on a JASCO J-720 or a Jobin Yvon Auto Dichrograph Mark IV spectropolarimeter using 1 mm quartz cells. Samples were in 20 mM NaPO₄ pH 8.0 and typically contained between 1 and 10 μ M protein. Protein concentrations were determined by quantitative amino acid analysis. Far-UV wavelength scans were recorded at 20°C from 190 to 250 nm as an average of four scans. Helical content was calculated by a CONTIN-fit of the experimental data (Provencher, 1992).

Yeast complementation studies

The MtGim α and MtGim β coding regions were subcloned into yeast expression vectors and complementation studies in yeast strains lacking *GIM* genes were performed essentially as described (Geissler *et al.*, 1998). Immunoprecipitations and Western blots were carried out using anti-Gim2, anti-MtGim α and anti-MtGim β antibodies also as described in Geissler *et al.* (1998) and above. Control immunoprecipitations were similarly performed in strains further deleted of *GIM2* to demonstrate the specificity of the interaction between the archaeal Gim proteins and the yeast proteins found in the anti-Gim2 immunoprecipitate.

Prevention of protein aggregation assays

Bovine rhodanese (100 μ M) (highly purified; Sigma), lysozyme (160 μ M) (Sigma) and *Tacidophilum* glucose dehydrogenase (200 μ M) (Sigma) were prepared in denaturing buffer (6 M guanidine hydrochloride, 20 mM

Tris pH 8.0, 100 mM NaCl, 1 mM MgCl₂ and 50 mM DTT in the case of lysozyme). The unfolded proteins were diluted 100-fold in buffer B (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM MgCl₂) alone as a reference for maximum aggregation or containing various amounts of MtGimC, individual MtGim subunits, or IgG as negative control proteins. Aggregation was then measured at 25°C over a period of 10 min by following the increase in light scattering at 320 nm. Alternatively, the effect of MtGimC on the thermally-induced aggregation of a 1 μ M solution of native rhodanese in buffer T was monitored at 320 nm in a thermostatted cuvette preheated to 42°C.

Formation of MtGimC–rhodanese complexes and protease sensitivity assays

Native rhodanese was made in buffer B to a concentration of 2 μ M. To prepare unfolded rhodanese and complexes between MtGimC and rhodanese, 200 μ M guanidine hydrochloride-denatured rhodanese was diluted 100-fold into buffer or in a solution containing 2 μ M MtGimC for 10 min and centrifuged to remove aggregates, respectively (incubated in buffer alone, most of the rhodanese can be pelleted). The protein samples (800 μ l) were concentrated to 50 μ l, separated by SEC (as described above) in buffer B, and relevant fractions were tested for protease sensitivity. This was done by incubation of the samples with proteinase K (Boehringer Mannheim; final concentration of 10 μ g/ml) on ice for 10 min. Reactions were stopped with 2 mM phenylmethylsulfonyl fluoride (PMSF) and analysed by Western blotting using the anti-rhodanese antibody.

Actin and rhodanese refolding experiments

Denatured, ³⁵S-labelled actin (D-actin) was prepared as described in Siegers *et al.* (1999). D-actin (~13 μ M) was diluted 100-fold either into buffer containing purified IgG, MtGimC or bovine testis TRiC (Frydman *et al.*, 1992) (final concentrations: IgG, 6 μ M; MtGimC, 2 μ M; TRiC, 0.3 μ M) and incubated at 30°C for 15 min. In some cases, these components were added following the incubation. Folding reactions were then carried out in the presence of 1 mM Mg-ATP for 1 h at 30°C, cleared of aggregated proteins by centrifugation (15 min at 14 000 r.p.m.), and analysed on 4.5% polyacrylamide, 80 mM MOPS-KOH pH 7.0 native gels containing 1 mM Mg-ATP (native PAGE). In a control reaction (50 μ l), 2 μ g of DNase I was incubated with the refolded actin for 5 min before analysis on the native gel. To demonstrate transfers of actin from MtGimC to GroEL or thermosome, MtGimC-actin complexes (2 μ M, prepared as described above) were incubated for 15 min with chaperonins (3 μ M) and analysed by native PAGE.

A 100 μ M stock of chemically denatured rhodanese was diluted 100-fold into refolding buffer (20 mM MOPS-KOH pH 7.4, 100 mM KCl, 2 mM Mg(OAc)₂ and 5 mM DTT) containing either 2 μ M MtGimC, 0.5 μ M GroEL or buffer alone. After a 15 min incubation at 25°C, GroEL (0.5 μ M final) was added to the refolding reaction devoid of chaperonin (where indicated) and incubated for another 10 min. Protein aggregates were then removed by centrifugation. At this point, aliquots from the reactions were analysed either by native PAGE (as above) and Western blotting using the anti-rhodanese antibody (data not shown) or by SEC. To the remaining supernatant, GroES (1 μ M final) and/or ATP (5 mM) were added to initiate rhodanese refolding by GroEL at 25°C. At the indicated time points, aliquots were assayed for rhodanese activity (Horowitz, 1995). Results are expressed as a percentage of a native rhodanese control.

Sequence alignments, structure predictions and phylogenetic analyses

Gim sequences were identified by BLAST searches of public databases at NCBI (<http://www.ncbi.nlm.nih.gov/blast/blast.cgi>) and the Sanger Centre (<http://www.sanger.ac.uk/>). Sequences were aligned hierarchically in MACAW (Schuler *et al.*, 1991). The existence of an internal repeat in α class subunits was detected by the occurrence of alternate alignments with near-identical scores to the optimal alignment. Secondary structure predictions were obtained from the JPred server (<http://cirrus.ebi.ac.uk:8081/submit.html>; Cuff *et al.*, 1998) and coiled coil predictions from the COILS server (http://www.ch.embnet.org/software/COILS_form.html; Lupas, 1996b). Phylogenetic analyses were performed by distance, using the PROTDIST and NEIGHBOR modules of Phylip 3.572 (<http://evolution.genetics.washington.edu/phylip.html>) and by maximum likelihood, using Puzzle 3.1 (<http://www.zi.biologie.uni-muenchen.de/~strimmer/puzzle.html>). All analyses were performed with and without inclusion of gapped positions, and 100 bootstrap replicates were calculated in each case. Figure 1B and C shows a subset of the sequences used in the computations.

Miscellaneous

Concentrations of purified proteins were determined using their predicted molar extinction coefficients. Protein lysate concentrations were estimated with the Bio-Rad assay using IgG as a standard. Molar concentrations refer to 6, 14 and 7 subunits for MtGimC, GroEL and GroES, respectively, and to monomers for actin, rhodanese, lysozyme and glucose dehydrogenase.

Acknowledgements

Special thanks to Dr R.Thauer for providing *M.thermoautotrophicum* Δ H cell pellets and to J.Pennings for his generous gift of genomic DNA from the hyperthermophile. We would also like to acknowledge Dr I.Gutshe for kindly giving us purified *T.acidophilum* α -thermosome, Drs K.Drickamer and R.Wallis for help with the ultracentrifugation experiments, and L.Weyher from the CD facility. M.R.L. and K.S. are holders of EMBO fellowships, and M.F. acknowledges a scholarship from the Rhodes Trust. C.M.D. holds an International Research Scholarship award from the Howard Hughes Medical Institute and acknowledges support from the Wellcome Trust as well as funding from the BBSRC, MRC and EPSRC to the Oxford Centre for Molecular Sciences.

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Received September 2, 1999; revised October 11, 1999;
accepted October 12, 1999