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Chaperonin filaments:
The archael cytoskeleton

Jonathan D. Trent,* Hiromi K. Kagawa, Takuro Yaai, Eric Olie,
and Nestor J. Zaluzec⁺

Center for Mechanistic Biology and Biotechnology

⁺Materials Science Division

Argonne National Laboratory

9700 S. Cass Avenue

Argonne, Illinois 60439

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

MASTER

tel: 630 252-3917

fax: 630 252-3788

email: Trent@ANL.CMB.bim.anl.gov

*Corresponding author:

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Summary

Chaperonins are multi-subunit double-ring complexes composed of 60-kDa proteins that are believed to mediate protein folding in vivo. The chaperonins in the hyperthermophilic archaeon *Sulfolobus shibatae* are composed of the organism's two most abundant proteins, which represent 4% of its total protein and have an intracellular concentration of ≥ 30 mg/ml. At concentrations of 1.0 mg/ml, purified chaperonin proteins aggregate to form ordered filaments. Filament formation, which requires Mg^{++} and nucleotide binding (not hydrolysis), occurs at physiological temperatures under conditions suggesting filaments may exist in vivo. If the estimated 4600 chaperonins per cell, formed filaments in vivo, they could create a matrix of filaments that would span the diameter of an average *S. shibatae* cell 100 times. Direct observations of unfixed, minimally treated cells by intermediate voltage electron microscopy (300 kV) revealed an intracellular network of filaments that resembles chaperonin filaments produced in vitro. The hypothesis that the intracellular network contains chaperonins is supported by immunogold analyses. We propose that chaperonin activity may be regulated in vivo by filament formation and that chaperonin filaments may serve a cytoskeleton-like function in archaea and perhaps in other prokaryotes.

Introduction:

The hyperthermophilic archaeon *Sulfolobus shibatae* lives in acidic geothermal hot springs and grows optimally at pH 3.0 and 83 °C (Grogan et al., 1990). Under all growth conditions, two of the most abundant proteins in this organism are its 60-kDa heat shock proteins, referred to as TF55 α and β (for review see Trent, 1996). Like heat shock proteins (HSPs) in other organisms, the synthesis of TF55 α and β increases significantly when *S. shibatae* is exposed to near-lethal temperatures (85-90 °C), while other protein synthesis decreases under these conditions. The increased TF55 synthesis correlates with an increase in the organism's ability to survive at lethal temperatures (90-95 °C) (Trent et al., 1990), and a significant role for the TF55 proteins in this enhanced survival is suggested by studies using amino acid analogs (Trent et al., 1994).

Enhanced survival after heat shock is observed in nearly all organisms and is referred to as acquired thermotolerance (Laszlo, 1988; Parsell and Lindquist, 1994; Trent 1996). In general acquired thermotolerance correlates with increased HSP synthesis, although the role of HSPs and the relative importance of other macromolecules remains controversial (Barnes et al., 1990; Bosch et al., 1988; Fisher et al., 1992; Li and Werb, 1982; Sanchez and Lindquist, 1990; VanBogelen et al., 1987). It has been proposed that a significant role of the HSPs in thermotolerance is to prevent the aggregation of intracellular proteins unfolded or damaged by high temperatures (Pelham, 1986), and indeed a number of HSPs have been shown to be able to bind and disaggregate proteins in vitro (Parsell et al., 1994; Skowyra et al., 1990; Ziemienowicz et al., 1993). This interaction of HSPs with unfolded proteins combined with their abundance in organisms under nonstress conditions has led to the hypothesis that some HSPs function as "molecular chaperones," i.e., they prevent inappropriate interactions between macromolecules in cells under normal conditions (Ellis et al., 1993; Georgopoulos and Welch, 1993; Hartl and Martin, 1995). The 60-kDa heat shock proteins (HSP60s) are such chaperones, believed to prevent inappropriate interactions between newly synthesized polypeptides and to thereby assist in protein folding (Frydman and Hartl, 1994; Gething and Sambrook, 1992; Hendrick and Hartl, 1993).

Sequence comparisons of all known HSP60s reveal two groups of these proteins, with high sequence homologies between proteins within each group and low (but unequivocal) homologies between proteins in the separate groups (Gupta, 1995; Kagawa et al., 1995). The HSP60s from bacteria, mitochondria, and chloroplasts form one group and those from archaea and the eukaryotic cytoplasm form the other. The proteins in both groups can be isolated from cells in association with high molecular mass complexes, known as chaperonins (Ellis et al., 1993; Hemmingsen, 1992), that appear as double-ring structures in the electron microscope (Phipps et al., 1993; Pushkin et al., 1982). In general, the chaperonins from the bacterial group have 14 subunits, 7 subunits in each of the two rings (Carazo et al., 1991; Chen et al., 1994), while those from archaea and the eukaryotic cytoplasm have 16-18 subunits, 8 or 9 subunits in each ring (Gao et al., 1992; Trent et al., 1991). Chaperonins in both groups share

functional features: they are ATPases, they are able to recognize and bind unfolded proteins, and they may influence the folding or refolding of some proteins (Frydman et al., 1994; Guagliardi et al., 1994; Martin et al., 1993; Trent et al., 1991).

Our understanding of how chaperonins influence protein folding comes primarily from in vitro experiments with the *Escherichia coli* chaperonin (GroEL). The current models of chaperonin-mediated protein folding suggest that an unfolded polypeptide enters the central cavity of the chaperonin; that folding occurs through either "caging" or "recycling" interactions within the chaperonin cavity; and that ATP hydrolysis and in some cases a co-chaperonin (GroES) is required for releasing the bound protein (Clarke, 1996). This model has been extrapolated with slight modifications to include the archaeal and eukaryotic-cytoplasmic chaperonins, with the implication that this protein-folding scheme is present in all organisms (Hartl and Martin, 1995; Frydman and Hartl, 1996).

Although chaperonin-mediated protein folding is now a widely accepted concept, it should be emphasized that the current model is based primarily on in vitro experiments conducted under conditions that are very different from those present in vivo. To expand our understanding of the in vivo function of chaperonins, we investigated the archaeal chaperonins, which have been referred to as "rosettasomes" (Trent, 1996). The archaeal chaperonins share features with both the bacterial chaperonins and the eukaryotic chaperonins, referred to as TCP1 chaperonins or "TriC" (Frydman et al. 1994). The constitutive proteins subunits of the archaeal chaperonins share sequence homology with those of the eukaryotic chaperonins, which has been the basis for placing them together in a separate group from the bacterial chaperonins. Like the bacterial chaperonins, however, the archaeal chaperonins are composed of one or two related subunits that in general are abundant, heat-inducible proteins. In contrast, the eukaryotic chaperonins are composed of up to eight related subunits that are not abundant in most cell types and are not heat shock proteins (Craig et al., 1993; Ursic and Culbertson, 1992).

To gain insights into the in vivo structure of archaeal chaperonins, we determined the intracellular concentrations of their constitutive proteins in the hyperthermophilic archaeon *S. shibatae* under normal

growth conditions. Using transmission electron microscopy (TEM), we observed that above a critical concentration purified chaperonins formed filaments in the presence of Mg^{++} and nucleotides. Filament formation occurred at physiological temperatures in both simple buffers and complex solutions made from cell extracts. In vitro observations suggested that chaperonin filament should exist in vivo, and calculations based on their abundance suggested they could create a significant internal structure. The cytostructure of *S. shibatae* cells was studied using TEM and immunogold techniques.

Results

The Intracellular Concentration of Chaperonin Proteins

We measured the amount of the two chaperonin proteins (TF55 α and β) in *S. shibatae* cells by comparing extracts from a predetermined number of cells with different amounts of purified chaperonin protein on polyacrylamide gels (Fig. 1). By this procedure we determined an average of 8.3×10^{-12} mg of TF55 per cell, which is approximately 4% of the cell's total protein. We transformed the amount of TF55 per cell into an intracellular concentration of TF55 by determining the average cell volume from calibrated scanning electron micrographs and the formula for a sphere. An average *S. shibatae* cell has a diameter of $0.8 \mu m$ and therefore an average volume of 2.7×10^{-13} ml, which gives an intracellular concentration for the chaperonin proteins of 30.7 mg/ml. (This is probably a conservative estimate, since we may have overestimated the cell volume by using the outside dimensions of cells and the formula for a sphere for the pleiomorphic *S. shibatae* cells).

Purified Chaperonins Form Filaments

To determine the effects of concentration on chaperonin structure, we purified chaperonins from *S. shibatae* cells grown at normal temperature ($75^{\circ}C$) and looked at samples of freshly prepared proteins by TEM using a standard negative staining procedure (Fig. 2). At a normal working concentration (0.1 mg/ml) for negative-stained protein samples, we observed the double-ring complexes characteristic of chaperonins (Fig.

2A). Since we had determined an intracellular protein concentration of ≥ 30 mg/ml, we prepared samples at higher protein concentrations, although above 2.0 mg/ml the proteins formed such thick layers on the sample grids they appeared completely black in TEM. At 0.5 mg/ml, however, we observed chains of chaperonins with the double rings stacked end to end (Fig. 2B). At 1.0 mg/ml, the chains were longer and frequently aligned side by side or intertwined to form an intricate network of filaments (Fig. 2C). These filaments formed at room temperature in a simple buffer (20 mM HEPES, pH 7.5; 10 mM KCl; 10 mM MgCl_2) and were stable at physiological temperature (75 °C) for up to 3 h. They did not form if Mg^{++} and K^+ were omitted from the buffer or if only K^+ was present (Fig. 2D & E), but did form if only Mg^{++} was present (Fig. 2F). Filaments could be dissociated by adding EDTA and reformed by adding excess Mg ion again, although this process could only be repeated a few times. Chaperonins stored for >48 h at -75 °C, -20 °C, 4 °C, or at room temperature, with or without Mg^{++} , lost their ability to form filaments.

Chaperonin Filament Formation Depends on Nucleotide Binding

Stored chaperonins that did not form filaments in the presence of Mg alone did form filaments in the presence of Mg^{++} and nucleotides (Fig. 3). (We suspect the freshly prepared chaperonins retained bound nucleotides and could therefore be induced to form filaments for a limited period of time). After a 1-h incubation at 75 °C, there was no change in the appearance of chaperonins in "aged" samples in the presence of Mg alone (Fig. 3A), whereas these same samples were transformed into an elaborate network of filaments in the presence of ATP/Mg (Fig. 3B). Filaments also formed in the presence of ADP/Mg (Fig. 3C), AMP-PNP/Mg (Fig. 3D), as well as GTP/Mg and $\text{ATP}\gamma\text{S}/\text{Mg}$ (data not shown), but these filaments were primarily isolated short chains and not the elaborate network seen with ATP/Mg. It was not determined why ATP/Mg was so much more effective in producing filaments than other nucleotides, but the observation that filaments formed in the presence of AMP-PNP, which is a non-hydrolyzable form of ATP, suggests the process depends on nucleotide binding rather than hydrolysis, and the superiority of ATP may reflect a higher binding affinity than other nucleotides. (We suspect chaperonin ATPase activity is involved in filament turnover, by analogy with actin).

We monitored the kinetics of chaperonin filament formation by a spectrophotometric procedure similar to the one used to monitor the polymerization of α and β tubulin (Wolff et al., 1996). We verified that the observed increase in light scattering at 350 nm correlated with filament formation using TEM, and then we monitored filament formation under a variety of conditions. Figure 4A shows filament formation at 75 °C in the presence of ATP/Mg, compared to ADP/Mg and the non-hydrolyzable AMP-PNP/Mg. As expected from TEM observations, the effects of ATP/Mg on filament formation were more pronounced than the effects of other nucleotides.

Both TEM and spectrophotometric observations indicated that chaperonin filaments formed spontaneously at physiological temperatures in a simple buffer containing Mg^{++} and nucleotides. To move closer to in vivo conditions, we monitored filament formation in a more physiological buffer made from cell extracts. This buffer was prepared by boiling extracts from lysed cell, pelleting the precipitates, and ultrafiltering the supernatant (see Experimental Procedures). This effectively removed or destroyed macromolecules (polyacrylamide gel electrophoresis indicated only small peptides remained), leaving salts and thermal-stable solutes. The extract was adjusted to pH 7.0, which is within the intracellular range reported for *Sulfolobus* spp. (Moll and Schäfer, 1988), and heated to 75 °C. At 75 °C, absorption (350 nm) gradually increased in the extract until chaperonin proteins were added to a final concentration of 1.0 mg/ml, which stabilized the extract until ATP/Mg was added and absorption increased in accord with what we had previously observed due to filament formation (Fig. 4B). The absence of filaments after incubation in the extract alone and their formation after the addition of ATP/Mg was confirmed by TEM observations.

Chaperonin Filaments Could Span the Diameter of a Cell 100 times

We calculated there are as many as 4635 chaperonins per cell. This value was derived from the amount of chaperonin protein per cell expressed in moles (8.3×10^{-9} μ g cpn protein/cell $\div$ 1.08×10^{12} μ g cpn/mole = 7.7×10^{-21} mol cpn/cell), and using Avagadro's number to translate this into the number of chaperonins (7.7×10^{-21} mol cpn/cell $\times$ 6.02×10^{23}

cpn/mol = 4635 cpn/cell). Using the reported size of the *Sulfolobus* chaperonin, 17.5 nm (Knapp et al., 1994), we then calculated that chaperonins could span the diameter of a typical *S. shibatae* cell 101 times. This assumes all 4635 chaperonins are associated with filaments and uses the average cell diameter (0.8 μ m) we measured from scanning electron micrographs. We reasoned that if such a mass of filaments exists in cells, it would form an intricate network that should be visible in the electron microscope under the appropriate conditions

Evidence that Chaperonin Filaments Exist In Vivo

In light of the chaperonin protein concentration in vivo, our observations of chaperonin filament formation in vitro, and our calculations above, we looked for filaments in *S. shibatae* cells grown at normal temperatures. Cells in late log phase growth were rapidly concentrated by centrifugation and placed on TEM sample grids. Cells attached to the grids were treated with a nonionic detergent (Triton X100) to remove their protein surface layer, treated with DNase to remove DNA polymers, and lightly stained with uranyl acetate to increase the contrast of cellular structures. (To avoid structural artifacts, cross-linkers such as gluteraldehyde or formaldehyde were not used). After this treatment, many cells remained essentially intact and despite these thick specimens, using intermediate voltage TEM (300 kV) we could distinguish an elaborate network of filaments in many cells (Fig. 5). Comparing low and high magnifications of the same cell, the network and some details of the filaments are visible (Fig. 5 A and A'). Comparing micrographs from different cells at approximately the same magnification, the similarity between individual filaments can be seen (Fig. 5 B-E). The average width of these intracellular filaments, 11 ± 1 nm, was nearly identical to that of the chaperonin filaments, 10.7 ± 0.6 nm. Furthermore, they have a definite periodic structure that is remarkably similar in appearance to the periodic structure of the chaperonin filaments formed in vitro (Fig. 6).

There are few other cellular structures known in archaea that could account for the intracellular filaments we observed. We considered the possibility that they are fragments of the *S. shibatae* surface layer (S-layer) or remnants of its chromatin. We rejected the S-layer hypothesis both because it is highly improbable that the continuous matrix of S-layer

proteins would fragment into filaments and because the unit-cell of the periodic structure of the S-layer (Baumeister and Lembcke, 1992; Kandler and König, 1993; Prüschenk and Baumeister, 1987), is different from the unit cell of the intracellular filaments we observed. (We verified this difference using TEM and atomic force microscopy of *S. shibatae* S-layers (unpublished observations). We also rejected the chromatin origin because of the difference in appearance between chromatin remnants (Bohrmann et al., 1990; Shioda et al., 1989) and the intracellular filaments we observed. We concluded from comparisons, as well as from our in vitro data, that the most plausible identity for the intracellular filaments we observed is that they are chaperonin filaments.

To support this hypothetical link between intracellular and chaperonin filaments, we used polyclonal antibodies against the chaperonin proteins and immunogold labeling to visualize the distribution of chaperonins in detergent-extracted cells (Fig. 7). The specificity of the labeling was indicated both by a preabsorption control, in which purified chaperonin was added to the antiserum used in the experiments (Fig. 7B), and by a pre-immune control, in which serum from the uninoculated animal was used (Fig. 7C). The specificity of the polyclonal antibody was determined by immunoblots of total *S. shibatae* proteins (not shown). Both the extent and pattern of labeling does support our hypothesis that the intracellular filaments consist of chaperonins. Since the procedure we used to prepare cells for labeling would be expected to remove most soluble proteins from cells, the extent of labeling we observed indicates that a considerable number of chaperonins are associated with detergent-resistant (i.e., insoluble) structures. These structures extend throughout the cells, as determined by stereo imaging. The pattern of immunogold labeling of cells is suggestive of filaments in some areas and similar to the pattern we observed in immunogold-labeling of the chaperonin filaments themselves.

Discussion:

The observation that the chaperonin protein concentration in *S. shibatae* cells is ≥ 30 mg/ml establishes an in vivo baseline for in vitro experiments.

It is not technically practical to visualize this high of a protein concentration by TEM, but the concentration-dependent aggregation of purified chaperonins into ordered filaments at 1.0 mg/ml, and the conditions under which these filaments form suggest that chaperonin filaments exist in vivo. This hypothesis is further supported by the similarity between chaperonin filaments and the array of filaments observed in *S. shibatae* cells, as well as by the distribution of chaperonin proteins in detergent-extracted cells. The hypothesis that chaperonins form filaments in vivo has many important implications for our understanding of how chaperonins may function in vivo and suggests alternate interpretations of experimental results, particularly results of genetic experiments with the eukaryotic chaperonin (TCP1).

One possible role for in vivo chaperonin filaments may be as a storage system for chaperonin activity. Current models suggest that chaperonin-mediated protein-folding requires access to the central cavity of the chaperonin through their apical region (Fenton et al., 1994; Hartl and Martin, 1995; Saibil, 1996). Since this region is buried in the filaments, chaperonin mediated protein-folding would be blocked by filament formation. Chaperonin activity may thus be regulated by regulating filament formation, which our data suggest may be mediated through Mg^{++} and nucleotide binding; conceivably, other divalent cations and nucleotide hydrolysis may also play a role. If filaments can dissociate into functional chaperonins, protein-binding and -folding capacity could be rapidly accessed in emergency situations (such as heat shock or other stresses) without the need for protein synthesis.

A second, more radical, implication for the hypothesis that chaperonins exist as filaments in vivo is that chaperonin filaments themselves are functional, providing a cytoskeleton-like structure in *S. shibatae* and perhaps other prokaryotes. Indeed it has been previously suggested that *Sulfolobus*, and other species of archaea that lack rigid cell walls, must have some kind of an internal "cytoskeleton," based on their ability to maintain an irregular shape in solution and to change their shape when attached to surfaces, and the gelling properties of their cytoplasm (Hixon and Searcy, 1993; Searcy and Hixon, 1991). The composition of this putative cytoskeleton has remained obscure, however, perhaps because efforts have been directed at finding archaeal homologues to the major

eukaryotic cytoskeletal proteins, actin and tubulin (Hixon and Searcy, 1993). The idea that the chaperonins form the basis for such a cytoskeleton is directly supported by their abundance and filament-forming ability, and indirectly supported by genetic studies with the phylogenetically related eukaryotic TCP1s.

It was previously reported that the two *S. shibatae* chaperonins are the organism's most abundant proteins (Kagawa et al. 1995), and we report here that they represent 4% of total proteins under normal growth conditions. The related proteins in other archaea are also abundant (see Kagawa et al. 1995). In *Pyrodictium* spp., for example, they represent about 6% of total proteins under normal growth conditions and reportedly reach 78% of total protein after heat shock (Phipps et al., 1991). (For comparison, actin and tubulin represent between 6% and 10% of total protein in most eukaryotes (Alberts et al., 1989)). We do not mean to imply that the abundance of archaeal chaperonins is proof that they are cytostructural. The fact that they are sufficiently abundant in vivo to produce a filamentous structure that could span the diameter of an average cell nearly 100 times, however, is consistent with a hypothesis that they function as building blocks for such a cytostructure. If they were not sufficiently abundant or if there were other *S. shibatae* proteins that were more abundant and could form filaments, their potential role as a cytostructure would be less plausible.

The role of archaeal chaperonins in protein folding under physiological conditions has not yet been demonstrated. The assumption that they are chaperonins is based primarily on their similarities to bacterial chaperonins, i.e., their double ring structure composed of 60-kDa heat shock proteins and their ability to recognize and bind unfolded proteins in vitro (Trent et al. 1991). Unlike the bacterial chaperonins that can influence the folding of a variety of proteins in vitro (Clarke, 1996), however, the archaeal and the phylogenetically related eukaryotic TCP1 chaperonins influence the folding of only a few proteins (Guagliardi et al. 1994; Kubota et al., 1995). The archaeal chaperonins have been reported to influence the folding of malic enzyme and alcohol and glucose dehydrogenases, but not at physiological temperatures (Guagliardi et al. 1994), and there are a number of thermophilic and mesophilic enzymes they do not fold at any temperature (unpublished information). The TCP1

chaperonins fold actin and tubulin in vitro (Gao et al., 1992; Yaffe et al., 1992; Sternlicht et al. 1993), but here too, there are many examples of cytoplasmic proteins in eukaryotes that they do not fold (Ellis, 1996; unpublished information). It is possible that archaeal and TCP1 chaperonins require some critical factors that are missing from these in vitro folding assays. It is also possible, however, that the role of chaperonins in vivo is not protein folding, but cytostructural.

There is evidence that the eukaryotic TCP1s play a direct role in the organization and function of the eukaryotic cytoskeleton. Mutational analyses in yeast indicate that TCP1s are essential proteins and that mutated TCP1s cause serious defects in cytoskeletal organization and cytoskeletal-related functions (Ursic and Culbertson, 1991; Vinh and Drubin, 1994; Ursic et al., 1994). TCP1 mutants show irregular distributions of actin and microtubules and are unable to segregate their nuclei properly, while overexpression of TCP1s can partially suppress actin mutations (Ursic et al., 1994). The intracellular distribution of TCP1 in yeast is primarily around the perimeter of the cell (Ursic et al. 1994), while in mammalian cells it is concentrated in the centrosome (Brown et al., 1996) or in medulla cells it is found associated with chromaffin granules (Creutz et al., 1995). These observations all suggest a direct role for TCP1 in the organization and function of the eukaryotic cytoskeleton rather than an indirect role, i.e., through mediating actin and tubulin protein folding.

It is not yet known if our observations on *S. shibatae* can be extrapolated to other prokaryotes, although there are some suggestive data. Internal structures have been observed in a variety of bacteria (reviewed by Bermudes et al., 1994), and the concept of a bacterial cytoskeleton (an "enzoskeleton") has been recently postulated (Norris et al., 1996). A tubulin-like protein (FtsZ) has been identified as a component of the enzoskeleton (Erickson, 1995), but other, more abundant proteins must also be involved. The chaperonin proteins are abundant in many bacteria. There is one report that a GroEL-related protein could be purified from spirochaetes using a protocol similar to the one used for tubulin purification (Munson et al., 1993), and two reports that mixtures of GroELS form filaments in vitro (Harris et al., 1994; Harris et al., 1995). Since many bacterial species have rigid cell walls (Koch, 1988), chaperonin filaments may play an organizational rather than a structural role in vivo.

The reason this structure has not been seen may be because it is fragile or ephemeral.

As a structural matrix, chaperonins may affect protein folding directly through interactions with folding intermediates or indirectly through macromolecular crowding (Garner and Burg, 1994). They may also be expected to affect other macromolecules, such as RNA (Georgellis et al., 1995; Herschlag, 1995), and other cellular processes such as transcription, translation, and enzyme channeling. As a cytoskeleton-like structure, they may control cytoplasmic organization on the most fundamental levels (Clegg, 1984; Wiggins, 1990). This hypothesis also suggests other possibilities for their role in the heat shock response and acquired thermotolerance, which may involve the structural stability of the cell itself. This hypothesis invites further investigation.

Experimental Procedures

Chaperonin purification

Chaperonins were purified from cells grown at 75 °C in standard yeast extract medium (Trent et al., 1990), opened by sonication (6 min x 2) in the presence of three volumes of HKM buffer (25 mM HEPES, pH 7.5, 10 mM KCl, and 10 mM MgCl₂), treated with DNase (0.25 U/ml, final), and centrifuged at 30,000 rpm for 30 min in a 50.2 Ti rotor (Beckman) before applying the supernatant to a DEAE-sepharose column, followed by a Mono-Q column (Pharmacia). Both columns were equilibrated in HKM buffer, and proteins were eluted in a 0 to 1.0 M NaCl gradient as previously described (Kagawa et al., 1995). The purified chaperonins were resuspended in 25 mM HEPES buffer (pH 7.5) and protein concentrations were determined by the DC Protein Assay system (BioRad) using BSA as a standard.

Polyacrylamide gel electrophoresis and spectrophotometry

Purified chaperonin subunits and total *S. shibatae* proteins were visualized by 10% polyacrylamide gel electrophoresis. Chaperonin proteins were purified as described above and total cellular proteins were extracted from specific numbers of actively growing cells. The number of cells was

determined by direct count using a haemocytometer (Neubauer), and the proteins were extracted by adding SDS-sample buffer (final conc. 10% glycerol, 100 mM DTT, 2% SDS, 50 mM Tris-HCl pH 6.8, 0.1% bromophenol blue) to the cell pellet produced by centrifugation (10,000 rpm, room temperature, 5 min, in a Hermle microfuge). The area and intensity of protein bands stained by Coomassie Brilliant Blue (R280) were quantified (area x intensity) by digitization using a flatbed, 10-bit, 1200-dpi scanner (Powerlook Pro scanner, UMAX) and the program NIH Image.

Spectrophotometry used a Cary 1G (Varian) with purified chaperonin in a simple buffer (5 mM HEPES, pH 7.0 or 7.5, 25 mM MgCl₂) or a complex buffer made by sonicating cells for 20 min, centrifuging (5,000 rpm, 10 min at 20 °C), boiling the supernatant for 10 min, re-centrifuging, and ultrafiltering the supernatant (10 kDa cutoff, centricon; Amicon). Adenosine triphosphate (ATP, Sigma) or 5'-adenylylimido diphosphate (AMP-PNP, Sigma) were used at final concentrations of 1 mM. Experiments were done at various temperatures regulated by the thermostated cell holder in the spectrophotometer.

Electron microscopy

Purified chaperonin samples were attached to lacy carbon grids with ultrathin Formvar (Ladd Scientific), stained with 2% uranyl acetate for 3 min, and air-dried. Cell samples (1 ml) in log phase growth at 75 °C were removed from medium by centrifugation (30 s, 12,000 rpm, tabletop centrifuge), washed in water, and resuspended in 50 µl HKM buffer (25 mM HEPES pH 7.5, 10 mM KCl, and 10 mM MgCl₂). An 8-µl sample of concentrated cells was placed on Formvar lacy grids treated with 2% Triton X100 for 3 min, washed in HKM buffer, treated with DNase (final conc. 0.1 U/ml, Promega) for 10 min, washed again in HKM buffer, stained in 2% uranyl acetate for 3 min, and then air-dried. All solutions were filtered (0.22 µm) and all procedures were done at room temperature.

Samples were viewed in a Philips EM420T or CM30T with LAB6 filaments at 80 to 300 kV or a JEOL 100CX with tungsten filament at 100 kV. No changes in the microstructure of samples were observed at the working resolution with electron doses of 1 to 200 electrons/Å². Micrographs were taken within this dose range at defoci of -200 to -800 nm with illumination-convergence angles of about 1 mrad and

scattering angle of 5 mrad using a room temperature, double-tilt, beryllium stage. Micrographs were digitized using a flatbed, 10-bit, 1200-dpi scanner (Powerlook Pro, UMAX), and data processing was done on Macintosh work stations with the programs NIH Image and Adobe Photoshop.

Immunogold labeling

Immunogold labeling followed established procedures (Hyatt, 1991). Cells were concentrated and attached to TEM grids, opened on grids in HP buffer (50 mM HEPES, 4% PEG 4000, 50 mM KCl, 5 mM MgCl) containing 2.5% Triton X-100 and 0.02 U/ μ l DNase (Promega); washed 3 times for 5 min in HP buffer; and fixed in 2% glutaraldehyde, 50 mM HEPES (pH 7.5) for 5 min. After three 5-min washes in PBS containing 0.1 M glycine (PBS-G), cells were treated with PBS containing 0.1% Tween-20, 5% dry milk powder, and 0.1 M glycine for 30 min and then were washed 3 times for 5 min with PBS-G. Cells were then soaked for 4 h in a humid chamber in 25 μ l of primary polyclonal antibody made in rabbits (diluted 1:1000 in PBS), preabsorbed primary antibody (diluted 1:1000 in PBS containing 1.0 mg/ml purified chaperonin), or preimmune antibody (diluted 1:1000 with PBS). Antibody-exposed cells were washed 3 x 5 min in PBS-G; soaked for 1 h in 25 μ l of secondary antibody (diluted 1:50), which was goat anti-rabbit IgG conjugated with 5 nm colloidal gold (EY Laboratories Inc.); washed 3 x 5 min with PBS-G; fixed as described above; washed for 15 min in filtered (0.22 μ m), double-distilled water; and air-dried.

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Figure Captions

Fig. 1: Abundance of chaperonin proteins in *S. shibatae* cells. The Coomassie-stained protein bands from 1.0 to 2.6 μg of "pure chaperonin protein" (left) was compared to "total proteins" extracted from $(1-4) \times 10^8$ cells (right) on a denaturing (SDS) polyacrylamide gel. The plot of the stained bands (area x intensity) vs. known amounts of pure protein was used to calculate the amount of chaperonin protein per cell (insert).

Fig. 2: Above a critical concentration freshly purified *S. shibatae* chaperonins form filaments at room temperature in the presence of Mg^{++} . A: purified chaperonin proteins in a HKM buffer (25 mM HEPES, pH 7.5; 10 mM KCl; 10 mM MgCl_2) appeared as double rings at concentrations of 0.1 mg/ml; B: rings and short chains were seen at 0.5 mg/ml; and C: long chains and filaments were present at 1.0 mg/ml. At chaperonin concentrations of 1.0 mg/ml filaments did not form in HEPES buffer alone (D), or when 10 mM KCl was added (E), but did form when 10 mM MgCl_2 was added (F).

Fig. 3: Chaperonins stored for >48 required Mg^{++} and nucleotides to form filaments at a physiological temperature. A: "aged" chaperonin (1 mg/ml) in HEPES buffer (pH 7.5) and 25 mM MgCl_2 after 1 h at 75 °C; B: the same chaperonin sample with 1 mM ATP added; C: with 1 mM ADP added; and D: with 1 mM AMP-PNP added.

Fig. 4: Chaperonin filament formation at 75 °C in the presence of Mg^{++} and nucleotides in different buffers measured by light scattering. A: changes in absorption (350 nm) of purified chaperonin (1.0 mg/ml) in HEPES buffer after the addition (inject) of 25 mM MgCl_2 and 1.0 mM ATP, ADP, or AMP-PNP; B: changes in absorption of purified chaperonin also occurred in a complex buffer made from cell extract (see Results) after the addition of MgCl and ATP (inject); the cell extract buffer itself (control) increased in absorption due to aggregation, which was suppressed by the addition of chaperonins but not after the chaperonins formed filaments. TEM analysis of samples before and after the addition of nucleotides

indicated that the increased absorption in chaperonin samples correlates with the presence of filaments.

Fig. 5: Micrographs of intracellular filaments in detergent-treated, unfixed *S. shibatae* cells lightly stained with uranyl acetate visualized using intermediate voltage TEM (300 kV). A low magnification (A) and high magnification (A') image of the same cell showing the distribution and periodic structure of the intracellular filaments (arrowheads). The thickness of the samples and the staining patterns made it difficult to visualize these structures, but similar filaments were seen in many cells (B-E).

Fig. 6: Chaperonin filaments formed in vitro (A) closely resembled the intracellular filaments when viewed at the same magnification (B).

Fig. 7: Immunogold labeling of detergent-treated *S. shibatae* cells with A: polyclonal antibodies against the chaperonin; B: the same antibodies pretreated with chaperonin; and C: with pre-immune serum. The 5-nm gold particles distributed on the support grid (visible as black spots) around the cells suggest that chaperonins were released during treatment. The abundance of gold particles remaining associated with the cells after the sample preparation, however, suggests that a large number of chaperonins are associated with an insoluble matrix. The pattern of gold particles in some regions is suggestive of filaments. Samples were not stained with uranyl acetate.

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Fig 1

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