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Purification of chaperonins from thermophilic bacteria and archaea

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Abstract

Chaperonins are among the most abundant proteins in thermophilic and hyperthermophilic microorganisms. A fast and efficient protocol has been designed to purify chaperonins from natural microbial sources on a milligram scale. The procedure takes advantage of the low isoelectric point and high molecular mass of chaperonins. A strong anion-exchange matrix in combination with gel permeation chromatography and separation on a high-resolution MonoQ column were used to purify four chaperonins from bacteria and archaea. We also show how the procedure for one of the hyperthermophilic chaperones can be easily scaled up. Pure chaperonins were characterized by two-dimensional polyacrylamide gel electrophoresis and high-performance liquid chromatography to establish heterogeneity. Pure chaperonins retain the characteristic double ring structure in electron microscopy and they bind unfolded proteins.

Keywords: Chaperonins; Proteins

1. Introduction

The need for highly stable enzymes in molecular biology and the biotechnology industry has created a great interest in proteins from thermophilic (50–80°C) and hyperthermophilic (>80°C) organisms [1]. Functional analysis has demonstrated the usefulness of these enzymes and proteins in many applications [2,3], but, the specific growth requirements of many hyperthermophilic organisms limit their large-scale cultivation in conventional fermentation systems. Consequently, direct purification of large quantities of these enzymes is difficult. The cloning and expression of thermophilic enzymes into mesophilic hosts, such as *E. coli*, has been often successful [3–5], but these enzymes do not always fold or assemble correctly [1,3]. This has led to an interest in molecular chaperones from these organisms which could assist in the proper folding and assembly of recombinant thermophilic enzymes [6,7]. Molecular chaperones have been shown to promote protein folding and assembly and prevent protein aggregation both in vivo [8,9] and in vitro [8]. One of the most important classes of molecular chaperones are the chaperonins (cpn60, hsp60, CCT). These ubiquitous proteins form large double-ring structures containing large solvent cavities. In bacteria this factor is composed of fourteen identical subunits [8,10] whereas in archaea, the 16- or 18-subunit complex is usually composed of two different, but sequence-related, subunits [11–14]. An exception to this has been described for the archaea *Methanococcus jan-*

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naschii, in which a single chaperonin gene was found [15]. The quaternary structure of this archaeal chaperonin is not yet known. The eukaryotic cytosolic chaperonin (CCT) shows even more complexity, with eight sequence-related subunits forming the 16-subunit chaperonin complex [16]. In bacteria, a co-chaperonin (cpn10) and ATP/Mg/K are often required for productive protein folding [8]. Archaeal chaperonins fold proteins in the presence of ATP/Mg/K, but it is not clear whether other protein cofactors participate in the folding process in vivo. Archaeal and bacterial chaperonins share only a limited sequence similarity [8,13–15] and show significant mechanistic differences [12].

The ability of chaperonins to protect enzymes from denaturation and aggregation, either by catalyzing productive folding and/or preventing irreversible unfolding and aggregation, has significantly increased interest in these proteins. Purified chaperonins could be used directly as enzyme stabilizers or, if co-expressed with the enzyme of interest in a mesophilic host, could direct the correct folding and assembly of the recombinant enzyme. However, because the primary function of chaperonins is to interact with proteins and peptides, the purification of chaperonins can be a challenging process.

We have developed a fast and effective milligram-scale purification procedure for chaperonins from a variety of organisms, including thermophiles and hyperthermophiles. The purification procedure includes cell lysis and protein extraction, centrifugation and three chromatographic steps separated by protein concentration, and can be completed in two days using the fast protein liquid chromatographic (FPLC) system. The procedure yields 25–50 mg of at least 95% pure chaperonin. In addition, a large scale version of the procedure yielded 0.7 g of pure chaperone from a hyperthermophile. Chaperonins purified by this procedure have been successfully crystallized and characterized biochemically [12,17,18].

2. Experimental

2.1. Reagents and buffers

Dephosphorylated bovine α-S1-casein, ATP and ADP were purchased from Sigma, human casein kinase II from Boehringer Mannhein, and ^{32}P-γ-ATP (>3000 Ci/mmol) from DuPont. Methanol was from Baxter. Buffer A: 50 m*M* Tris–HCl pH 7.5, 1 m*M* dithiothreitol (DTT), 1 m*M* EDTA; buffer B: 50 m*M* Tris–HCl pH 7.5, 1 m*M* DTT, 1 m*M* EDTA, 500 m*M* NaCl; buffer C: 50 m*M* Tris–HCl pH 7.5, 1 m*M* DTT, 1 m*M* EDTA, 250 m*M* NaCl and 20% (v/v) methanol; Triton lysis buffer: 50 m*M* Tris–HCl pH 7.5, 0.5% Triton X-100 (v/v); buffer D: 50 m*M* $NaKHPO_4$ pH 6.6, 100 m*M* Na_2SO_4, 2 m*M* NaN_3, 5 m*M* $MgCl_2$. All reagents were analytical grade.

2.2. Bacterial strains

Thermus thermophilius HB8 (ATCC 27634) was grown at 75°C in a modified Castenholtz TYE medium [28] that contained five parts of 1% TYE medium [10 g Tryptone (Difco 0123) and 10 g yeast extract dissolved in 1 l of distilled water]. *Sulfolobus shibatae* (DSM strain 5389) was obtained from Dr. J.D. Trent and was grown at 80°C in liquid medium (containing yeast extract and Brock's salts) in a 150 l fermentor as described previously [13]. *Thermococcus litoralis* (DSM 5473) was grown at 85°C in the absence of sulfur in a 600 l fermentor as described previously [29].

2.3. Chaperonin purification

This purification protocol was developed for the chaperonins from *Sulfolobus shibatae* (TF55, archaeosome, rosetteasome) [16], *Thermococcus litoralis* (thermosome) and *Thermus thermophilus* (hsp60); with minor changes, it can be used for other chaperonins from archaeal and bacterial sources. 50 g of frozen cell paste was suspended in 100 ml of buffer A and the pH was adjusted to 7.5 with 0.1 *M* NaOH. In the case of *S. shibatae*, an equal volume of Triton lysis buffer was added to the cell suspension. The solution was mixed gently and homogenized by sonication on ice (5 times, 30 s). The cell extract was cleared by centrifugation (12 000 rpm, 30 min, GSA rotor from Sorval) and applied (5 ml/min) to a freshly regenerated FastQ column (30×2.3 cm) that was pre-equilibrated with buffer A. The column was washed with 500 ml of buffer A (5 ml/min) and

proteins were eluted with a linear gradient of 0 to 500 m*M* NaCl (1 l).

Fractions containing chaperonin were identified by native 4–15% polyacrylamide gel electrophoresis with standards using the PHAST minigel system from Pharmacia. The chaperonins elute between 60–220 m*M* NaCl for *S. shibatae* chaperonin, and 140–220 m*M* NaCl for *T. thermophilus* chaperonin. Generally, the concentration of NaCl needed to elute chaperonin from the FastQ column reflects the p*I* of the chaperonin; more acidic chaperonins elute at higher NaCl concentrations. Fractions containing chaperonin were concentrated in an Amicon nitrogen-pressurized stirred cell using a YM-100 membrane (Difco) to a final volume of 10 ml and applied directly to a gel filtration column (Sephacryl 300, 150×2.5 cm) pre-equilibrated with buffer C. We routinely loaded onto the column 50–200 mg of protein. The column was run at 0.5 ml/min. Chaperonin eluted in a sharp peak near the void volume. Fractions containing chaperonin were identified by native gel electrophoresis, diluted with one volume of buffer A, and applied to a MonoQ 16/10 column pre-equilibrated with buffer A. The chaperonin was eluted with a linear 200–350 m*M* NaCl gradient. Conditions were optimized to partially separate the two different conformers of the archaeosome [12]. Native (4–15%) and sodium dodecyl sulfate (SDS) (12.5%) polyacrylamide gels were run with standards to evaluate purity. Finally, pure chaperonin fractions were concentrated using centrifugal concentrators (Filtron).

T. litoralis chaperonin was also purified on preparative scale. Cell extracts were prepared from 800 g (wet weight) of frozen cells under strictly anaerobic conditions as described previously [23]. All chromatography columns were operated using a FPLC system (Pharmacia LKB, Piscataway, NJ, USA). The purification procedure was the same as for the purification of *T. litoralis* formaldehyde ferredoxin oxidoreductase [23] up to and including the first chromatography step which used a column (21×7.5 cm) of DEAE Sepharose Fast Flow (Pharmacia LKB). Chaperonin eluted at 220–280 m*M* NaCl using a gradient (9 l) from 0 to 500 m*M* NaCl in buffer A. Fractions (100 ml) from this column containing chaperonin as determined by SDS gel electrophoresis were combined (800 ml) and concentrated to approximately 30 ml by ultrafiltration (Amicon PM-30). The concentrated sample of chaperonin was then applied to a column (60×6 cm) of Superdex 200 (Pharmacia LKB) equilibrated at 4 ml/min with buffer containing 200 m*M* NaCl. Fractions (30 ml) were collected and those containing chaperonin were combined yielding approximately 700 mg of the purified protein. Chaperonin from *Pyrococcus furiosus* (DSM 3638) was obtained by a procedure similar to that described for the *T. litoralis* protein.

2.4. Determination of protein concentration and purity

The gene sequences of chaperonins from *E. coli* [19], *T. thermophilus* [20], the α and β subunit from *T. litoralis* [25], and the α and β subunit from *S. shibatae* [13] are known. Protein concentration was determined by spectroscopy using extinction coefficients calculated from the amino acid sequence of the chaperonins [21]: *E. coli* GroEL, 9320 M^{-1} cm^{-1}; *T. thermophilus* hsp60, 7680 M^{-1} cm^{-1}; *T. litoralis* α and β subunit 9200 M^{-1} cm^{-1} and 7920 M^{-1} cm^{-1}, respectively; and *S. shibatae* α and β subunit 9080 M^{-1} cm^{-1} and 32 550 M^{-1} cm^{-1}, respectively. The extinction coefficients for the oligomeric chaperonins from *T. litoralis* and *S. shibatae* were calculated separately, as they comprise both α and β subunits; they were found to be 136 960 M^{-1} cm^{-1} and 374 670 M^{-1} cm^{-1}, respectively. The concentration of *P. furiosus* chaperonin was estimated by the protein assay from Pierce using *S. sulfolobus* chaperonin as a standard. Purified thermophilic proteins were stored at room temperature at protein concentrations higher than 50 mg/ml or at −25°C in the presence of 30% glycerol. Protein purity was determined by HPLC gel filtration chromatography and by two-dimensional (2D) gel electrophoresis and silver staining. Protein purity was confirmed by N-terminal sequencing of bacterial chaperonins. The N-terminus of the archaeal chaperonins was blocked and these proteins were identified by sequencing of tryptic and chymotryptic fragments (W.M. Keck Foundation, Biotechnology Resource Laboratory, Yale University). Chaperonin preparations were at least 95% pure, were active in ATP hydrolysis, protein binding and protein folding

according to the assays previously described [6,18] and, as described below, they all produced excellent electron micrographs. Chaperonins purified using the procedures described above were crystallized and some of the crystals diffracted X-rays to high resolution [22]. The overall yield of chaperonin was usually between 0.5 and 1.0 mg of chaperonin per g of wet cell paste for thermophilic bacteria and archaea (Table 1).

2.5. Two-dimensional gel electrophoresis

2D gel electrophoresis was done as described previously [23]. Briefly, protein samples were mixed with an equal volume of 9 *M* urea, 4% (v/v) Nonidet P40, 2% 2-mercaptoethanol and 2% ampholytes (LKB pH 9–10). First-dimension isoelectric focusing (IEF) was done using 40-cm rod gels containing 50% pH 3–10 and 50% pH 5–7 ampholytes from Bio-Rad. After IEF, the tube gels were equilibrated in a buffer containing SDS. Second-dimension SDS–polyacrylamide gel electrophoresis (PAGE) was run in slab gels containing a 10–17% linear gradient of acrylamide. Gels were fixed in 50% (v/v) ethanol with 0.1% formaldehyde and 1% acetic acid and stained with silver.

2.6. Electron microscopy

Specimens dissolved in 20 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.5, were applied to a 400-mesh glow-discharged carbon grid and stained with 1% uranyl acetate. In order to obtain the best contrast an ultra thin (approx. 10 Å) carbon film was laid over a holey carbon film to provide as thin a substrate as possible.

Table 1
Summary of total yield of hsp60 from *T. Thermophilus* (46.2 g of cells)

	Volume (ml)	A_{280}	hsp60 (mg)
Crude extract	210	672	~92[a]
FastQ	130	114	81.5
S-300	35	13.6	74.2
MonoQ	44	8.1	62.3

[a] Estimated from gel electrophoresis.

Electron micrographs were obtained using a Philips CM10 electron microscope at 100 kV and at an electron optical magnification of 52 000.

3. Results and discussion

Using the described protocols, four different chaperonins – *E. coli* GroEL, hsp60 from *T. thermophilus*, the thermosome from *T. litoralis* and the archaeosome from *S. shibatae* – were purified to homogeneity on a milligram scale (Table 1), and the *T. litoralis* protein was obtained on a gram scale. The best characterized of these, GroEL, was used as a reference protein both in method development and subsequent biochemical and structural analyses, since a similar purification scheme was used to obtain *E. coli* GroEL from an overproducing strain for crystallization experiments [13]. The purification procedure was designed to exploit several properties of the chaperonins. These proteins are rather acidic, with an isoelectric point (p*I*) in the pH range of 4.7–5.4, and they bind well to a strong ion-exchange quaternary amine-type matrix (FastQ or MonoQ) (Fig. 1). Chaperonins form double-ring structures that are very large, from $800 \cdot 10^3$ to $1100 \cdot 10^3$ in molecular mass. As chaperonins are one of the most abundant large soluble proteins in the cell types under study, gel permeation chromatography was extremely effective in separating them from most of the other cellular proteins (Figs. 1 and 2). In order to remove proteins and peptides bound to the chaperonin, permeation chromatography was carried out in the presence of 20% methanol [24]. The final step, in which the highly enriched chaperonin fraction is applied to a high-resolution MonoQ column, provides chaperonin which is >99% pure. The size of the chaperonin complexes represents a potential problem, as the slower diffusion of such large particles can affect separation on high-efficiency columns. In our hands MonoQ columns appear to perform well; although the flow-rates used were 20–30% lower than that suggested for smaller proteins.

Electron microscopy of the bacterial chaperonins GroEL and *T. thermophilus* hsp60 show that they both have 7-fold symmetry, with 14 subunits in the chaperonin complex. The presence of a single band

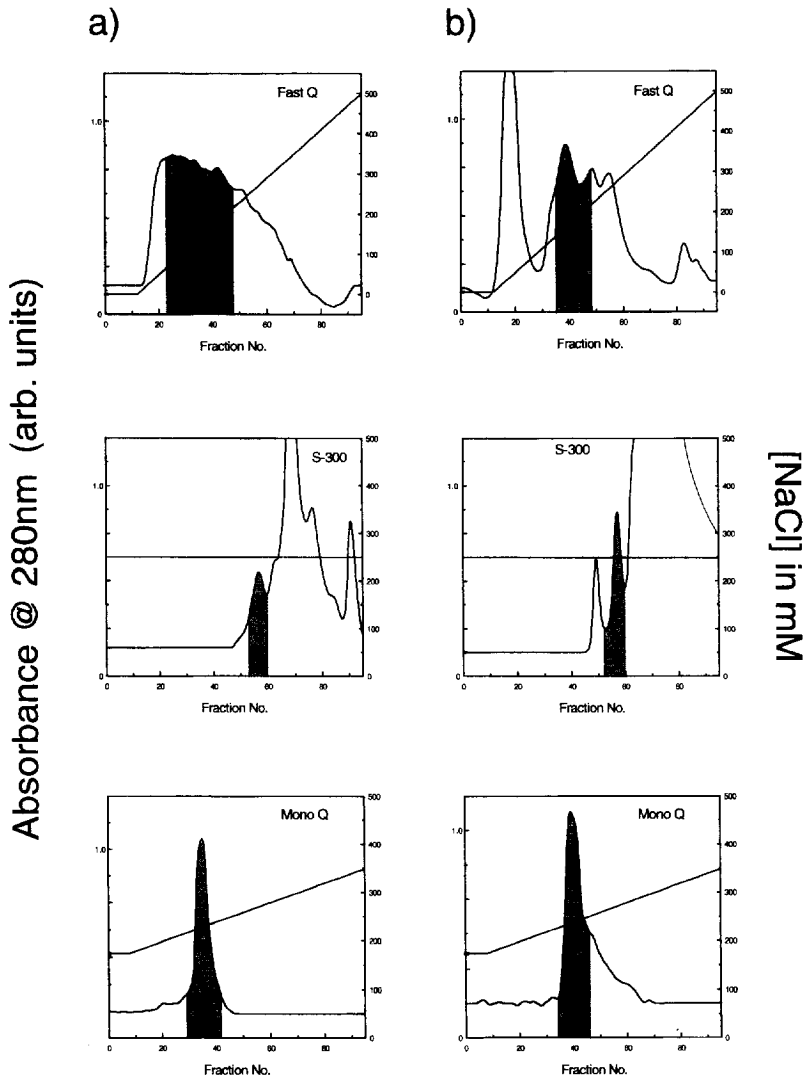

Fig. 1. Summary of the chromatographic steps in chaperonin purification. (a) Purification of *S. shibatae* on FastQ, Sephacryl S-300 and MonoQ columns, (b) purification of *T. thermophilus* on FastQ, Sephacryl 300 and MonoQ columns. Absorbance at 280 nm and concentration of NaCl is plotted for each chromatographic separation. Bar indicates fractions in each step that contained chaperonin and were pooled.

after SDS-PAGE indicates that the complex is homo-oligomeric, and this was confirmed by N-terminal sequencing. In contrast, the *T. litoralis* thermosome and the *S. shibatae* archaeosome show 8- and 9-fold symmetry, respectively, with 16 and 18 subunits present in the oligomeric complex. 2D gel electrophoresis of the archaeosome and the subsequent sequencing of chymotryptic fragments confirmed that the chaperonin complex from *S. shibatae* contains two sequence-related, but different subunits. Characterization of this chaperonin is described in detail elsewhere [13]. Less is known about the *T. litoralis* chaperonin complex. 2D gel electrophoresis indicates the presence of four species having different charges but with similar molecular masses; whether these are different subunits is unknown at present. Recently, two genes coding for the chaperonin-like proteins have been cloned from *T. litoralis* [25], and studies

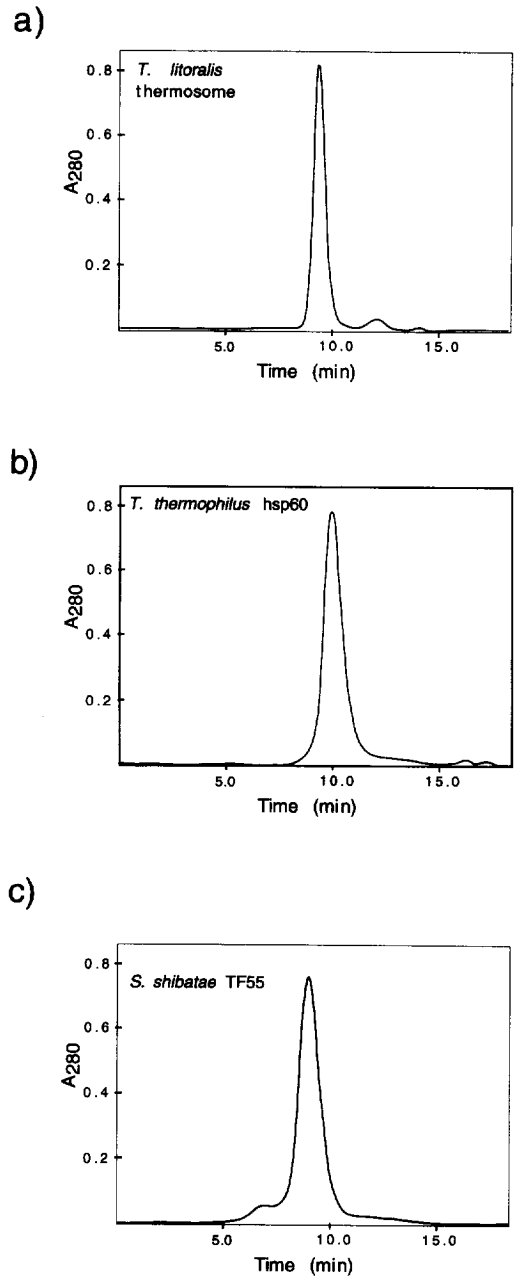

Fig. 2. Separation of purified hsp60 chaperonin from (a) *T. litoralis*, (b) *T. thermophilus* and (c) *S. Shibatae* on TSK-4000 gel permeation chromatography using HPLC in buffer D. The samples were run at a flow-rate 1.0 ml/min, and absorbance at 280 nm was plotted versus fraction number.

are in progress to determine if both of the encoded proteins are present in the purified complex.

Purified chaperonins were further characterized by 2D gel electrophoresis. While the results from these studies confirmed the purity of the preparations, it was also clear from the 2D electrophoresis gels that several isoforms are present in the chaperonin preparations, the archaeal chaperonins showing significantly higher heterogeneity than the bacterial ones. This could be explained by phosphorylation, as suggested for *S. solfataricus* [26], or by deamidation occurring at the high growth temperatures of these organisms. Whether the presence of isoforms in vivo is an important aspect of chaperonin function is not known at present. For example, the α and β subunits purified from *S. shibatae* can be phosphorylated in vitro but the extent that this occurs in vivo has yet to be established [12]. Comigration studies of the proteins purified in this study together with the chaperonin from the hyperthermophile *P. furiosis* show the extent of variation in the chaperonin family of proteins (Fig. 3). The molecular masses of the different chaperonin subunits are quite similar ranging between $57 \cdot 10^3$ and $61 \cdot 10^3$. The mobility of the subunits in the first dimension (IEF) accurately reflects the p*I* calculated directly from the sequence. Therefore, the proteins from *E. coli*, *T. litoralis* and *P. furiosis*, with calculated p*I* values of 4.74, 4.8 and 4.75, respectively, migrate to almost identical positions on the gel. The chaperonin from *S. shibatae* was the least acidic of the proteins examined (p*I* 5.3) while that from *T. thermophilus* was intermediate (p*I* 5.1).

The purification of functional chaperonins from thermophilic bacteria and archaea is particularly relevant to the issue of protein thermostability, since chaperonins were originally discovered as a response to heat shock. To date, most mechanistic studies have centered on GroEL and related cofactors, where the ability of the chaperonin to protect and refold chemically denatured protein substrates has been examined in depth [8,27]. The use of thermophilic analogs of GroEL such as those described herein will allow the investigation of the protection and refolding of thermally denatured protein substrates. Although much is known about GroEL structure and function, attempts to fully characterize the eukaryotic cytosolic chaperonin (CCT) have proved difficult,

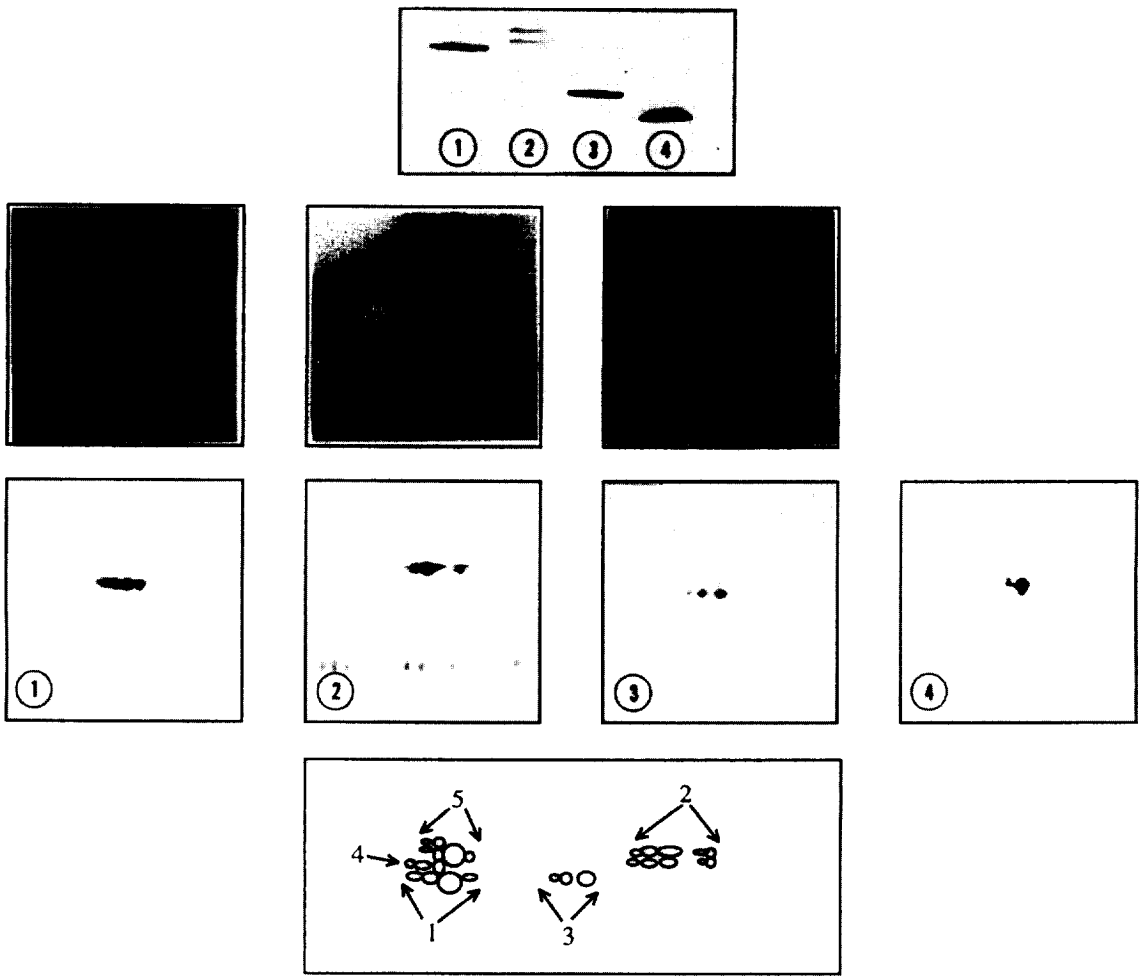

Fig. 3. Electron microscopic images and polyacrylamide electrophoresis of purified chaperonins from bacterial and archaeal sources. Top panel shows separation of chaperonins on 4–15% polyacrylamide gel under native conditions. The middle panel shows 2D gel electrophoresis and corresponding electron microscopic images of four chaperonins from bacterial and archaeal sources. The archaeosome sample was run on a 2D electrophoresis gel, with charge standards shown as a line of protein spots below the chaperonin sample. The bottom panel shows the relative position of five different chaperonins on 2D gel electrophoresis. The observed charge and mass polymorphism of thermophilic archaeal chaperonins is contrasted with more homogenous preparations of bacterial chaperonins derived from mesophilic and thermophilic sources. The numbering scheme for chaperonins is as follows: (1) *T. litoralis*, (2) *S. shibatae*, (3) *T. thermophilus*, (4) *E. coli* and (5) *P. furiosus*.

presumably due to the subunit complexity of these molecules. The archaeal chaperonins show a high degree of homology to CCT but they contain only one or two related subunits. These chaperonins therefore provide a simpler system by which to examine the structure and function of CCT in chaperonin-mediated protein folding.

The high yield afforded by the purification protocols described herein has allowed many aspects of thermophilic chaperonins to be examined. As examples, crystals grown from these preparations have diffracted X-rays to high resolution [22], and small-angle neutron scattering experiments to determine the relationship of the chaperonin to its protein substrate have been carried out [17]. In addition, biochemical studies have shown that the chaperonins interact with many different protein substrates, dependent on temperature, denaturation state and the

presence of cofactors. Further studies will elucidate the exact role of chaperonins in thermotolerance and protein folding and could provide mechanisms by which important enzymes can be stabilized both in vivo and in vitro.

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References

[1] I. Lasa, J. Berenguer, Microbiologia Sem. 9 (1993) 77.
[2] D.R. Engelke, A. Krikos, M.E. Bruck, D. Ginsburg, Anal. Biochem. 191 (1990) 396.
[3] K.A. Laderman, K. Asada, T. Uemori, H. Mukai, Y. Taguchi, I. Kato, C.B. Anifinsen, J. Biol. Chem. 268 (1993) 24402.
[4] N. Benachenhou-Lahfa, B. Lbedan, P. Forterre, Gene 140 (1994) 17.
[5] J.M. Muir, R.J.M. Russel, D.W. Hough, M.J. Danson, Protein Eng. 8 (1995) 583.
[6] A. Guagliardi, L. Cherchia, S. Bartolucci, M. Rossi, Protein Sci. 3 (1994) 1436.
[7] H. Taguchi, J. Konishi, N. Ishii, M. Yoshida, J. Biol. Chem. 266 (1991) 22411.
[8] M. Mayhew, A.C.R. daSilva, J. Martin, H. Erdjument-Bromage, P. Tempst, F.-U. Hartl, Nature 379 (1996) 420.
[9] O. Fayet, T. Ziegelhoffer, C. Georgopoulos, J. Bacteriol. 171 (1989) 1379.
[10] K. Braig, Z. Otwinowski, R. Hegde, D.C. Boisvert, A. Joachimiak, A.L. Horwich, P.B. Sigler, Nature 371 (1994) 578.
[11] B.M. Phipps, A. Hoffmann, K.O. Stetter, W. Baumeister, EMBO J. 10 (1991) 1711.
[12] E. Quaite-Randall, J.D. Trent, B. Josephs, A. Joachimiak, J. Biol. Chem. 270 (1995) 28818.
[13] H.K. Kagawa, J. Osipiuk, N. Maltsev, R. Overbeek, E. Quaite-Randall, A. Joachimiak, J.D. Trent, J. Mol. Biol. 253 (1995) 712.
[14] T. Waldmann, M. Nitsch, M. Klumpp, W. Baumeister, FEBS Lett. 376 (1995) 67.
[15] C.J. Bult et al., Science, 273 (1996) 1058.
[16] A.L. Horwich, K.R. Willison, Phil. Trans. R. Soc. Lond. B 339 (1993) 313.
[17] P. Thiyagarajan, S.J. Henderson, A. Joachimiak, Structure 4 (1995) 79.
[18] A. Joachimiak, K. Knowlton, E. Quaite-Randall, FASEB J. 9 (1995) 52.
[19] S.M. Hemmingsen, C. Wollford, S.M. Van der Vies, K. Tilly, D.T. Dennis, C.P. Georgopoulos, R.W. Hendrix, R.J. Ellis, Nature 333 (1988) 330.
[20] M. Erbeznik and A. Joachimiak, Genbank Accession Number TA 1060 U29483.
[21] S.C. Gill, P.H. von Hippel, Anal. Biochem. 182 (1989) 319.
[22] A. Joachimiak, M. Sriram R-G. Zhang, M.W.W. Adams and J.D. Trent, 6th International Conference on Crystallization of Biological Macromolecules, Hiroshima, Japan, 1995.
[23] C.S. Giometti, S.L. Tollaksen, S. Mukund, Z.H. Zhou, K. Ma, M. Xuhong, M.W.W. Adams, J. Chromatogr. A 698 (1994) 341.
[24] G. Lorimer, personal communication.
[25] J. Osipiuk, M. Adams and A. Joachimiak, Genome Sequence Data Base (L78246 and S1113612).
[26] S. Knapp, I. Schmidt-Krey, H. Herbert, T. Bergman, H. Jornvall, R. Ladenstein, J. Mol. Biol. 242 (1994) 397.
[27] J.S. Weissman, Y. Kashi, W.A. Fenton, A.L. Horwich, Cell 78 (1994) 693.
[28] R.W. Castenholz, Bacteriol. Rev. 33 (1969) 476–504.
[29] S. Mukund, M.W.W. Adams, J. Biol. Chem. 268 (1993) 13592–13600.