ACCELERATED PUBLICATION

Nucleotide-dependent protein folding in the type II chaperonin from the mesophilic archaeon *Methanococcus maripaludis*

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We report the characterization of the first chaperonin (Mm-cpn) from a mesophilic archaeon, *Methanococcus maripaludis*. The single gene was cloned from genomic DNA and expressed in *Escherichia coli* to produce a recombinant protein of 543 amino acids. In contrast with other known archaeal chaperonins, Mm-cpn is fully functional in all respects under physiological conditions of 37 °C. The complex has Mg²⁺-dependent ATPase activity and can

prevent the aggregation of citrate synthase. It promotes a high-yield refolding of guanidinium-chloride-denatured rhodanese in a nucleotide-dependent manner. ATP binding is sufficient to effect folding, but ATP hydrolysis is not essential.

Key words: chaperonin, citrate synthase, protein folding, rhodanese, thermosome.

INTRODUCTION

Chaperonins are ubiquitous in all domains of life, and function as ATP-driven, protein-folding machines [1]. They can be subdivided into two classes on the basis of their evolutionary origin. Bacterial GroEL and the chaperonins of the endosymbiont-derived organelles in eukaryotes belong to the type I class, whereas archaeal thermosomes and the eukaryotic CCT [cytosolic chaperonin containing T complex polypeptide-1 (TCP-1)] are members of the type II class. Regardless of their origin, all chaperonins are multimeric double-ring cylinders composed of approx. 60 kDa subunits [2,3]. Type II chaperonins form octa- or nona-meric rings comprising one to three (thermosome) or eight (CCT) different subunits [1]. Within each subunit, a globular apical domain, where unfolded substrate protein binds, is connected via a small intermediate domain to the equatorial domain, which binds nucleotide and forms the interface between the two rings. The folding of substrate proteins by type I and II chaperonins occurs in the central cavity formed by each ring [4,5]. This cavity can be sealed off from the external environment by a lid, allowing the thus-isolated substrate protein to fold more efficiently. The lid function for type I chaperonins is provided by a separate co-chaperonin protein (GroES in bacteria) [6]. In contrast, a unique structural feature, termed the helical protrusion, might act as a built-in lid to seal off the central cavity of type II chaperonins during folding [3,7].

Our understanding of chaperonin function comes mainly from experiments performed with bacterial GroEL. Several difficulties inherent in working with type II chaperonins have resulted in a dearth of biochemical data on these machines. Eukaryotic CCT rings consist of eight subunits, each being the product of a different gene [1,8]. This makes the generation of a recombinant system that is amenable to mutagenesis difficult. Moreover, CCT is susceptible to dissociation and proteolysis [9]. As a result, several laboratories have turned to archaeal chaperonins as potential model

systems for type II chaperonins [3,5,7,10–14]. However, the archaeal chaperonins studied to date have provided their own unique challenges. When expressed recombinantly in *Escherichia coli*, multi-subunit archaeal chaperonins may not assemble into authentic particles with correct subunit composition, whereas expression of a single subunit type may lead to insoluble or inactive assembled protein [10,11]. Furthermore, all of the archaeal chaperonins studied to date have come from thermophilic sources. As such, many exhibit limited activity *in vitro*, especially at temperatures below their significantly elevated growth optima [1,10–12,14]. The few archaeal chaperonins that can promote refolding of substrates [5,13] do so only at these elevated temperatures, which prevents the use of traditional mesophilic model substrate proteins for folding assays. Thus a model chaperonin system that retains full function at ambient temperatures would be highly advantageous, but has been unavailable until now. In the present study, we describe the cloning, expression and biochemical characterization of the chaperonin from the mesophilic archaeon *Methanococcus maripaludis* (Mm-cpn) [15]. We report that Mm-cpn possesses full chaperonin activity at ambient temperatures, and can promote the refolding of rhodanese, a long-standing model substrate protein in protein folding studies [4,16], thereby allowing direct comparison with the GroEL mechanism of action. We show that ATP binding is sufficient to exert the chaperonin function, as rhodanese refolding proceeds in the presence of ATP or adenosine 5'-[β , γ -imido]triphosphate (p[NH]ppA). Its features designate Mm-cpn as an attractive model system for the study of type II chaperonins.

EXPERIMENTAL

Cloning of the Mm-cpn gene

Genomic DNA from *M. maripaludis* strain LL and a genomic library of *M. maripaludis* in λ -DashII were kindly provided

Abbreviations used: TCP-1, T complex polypeptide-1; CCT, chaperonin containing TCP-1; Mm-cpn, *Methanococcus maripaludis* chaperonin; p[NH]ppA, adenosine 5'-[β , γ -imido]triphosphate; GdmCl, guanidinium chloride; DTT, dithiothreitol.

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The nucleotide sequence data reported for the *Methanococcus maripaludis* chaperonin gene will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AY099402.

by Dr John Leigh (Department of Microbiology, University of Washington, Seattle, WA, U.S.A.). A series of degenerate oligonucleotide primers (MWG), designed on the basis of sequence alignments of archaeal chaperonin sequences, were used in PCR reactions with *M. maripaludis* genomic DNA as the template. This enabled the cloning of approx. 90 % of the internal coding sequence for Mm-cpn. A genomic *M. maripaludis* library was screened by plaque hybridization on to nylon filters, in order to determine the missing 5' and 3' sequences. A 725 bp digoxigenin-labelled probe (Roche–Boehringer–Mannheim), generated by PCR, was used for hybridization. For Southern blotting, *M. maripaludis* genomic DNA was digested with *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I and *Pst*I restriction endonucleases, separated on an agarose gel and blotted overnight on to Nytran membrane (Schleicher and Schuell, Dassel, Germany) by capillary transfer [17].

Expression and purification of Mm-cpn

The Mm-cpn gene was amplified by PCR with primers having flanking *Nde*I and *Bam*HI restriction sites. After ligation into a pET30b vector (Novagen, Madison, WI, U.S.A.), the construct was expressed in *E. coli* BL21(DE3) cells (Novagen) by induction with 1 mM isopropyl β -D-thiogalactoside. After 4 h, cells were harvested by centrifugation and pellets were resuspended in 150 ml of lysis buffer [30 mM Tris/HCl (pH 7.5)/100 mM NaCl/5 mM MgCl₂/15 % (v/v) ethanol/0.1 % (w/v) Triton X-100/1 mM PMSF/200 μ g $\cdot$ ml⁻¹ lysozyme]. The suspension was sonicated and the lysate was centrifuged three times (12 000 g; 20 min each spin) to pellet debris. The supernatant was applied to a Mono Q 16/10 column (Pharmacia, Piscataway, NJ, U.S.A.) pre-equilibrated with buffer A [30 mM Tris/HCl (pH 7.5)/100 mM NaCl/5 mM MgCl₂]. Elution was performed with a linear gradient of 0–45 % buffer B [30 mM Tris/HCl (pH 7.5)/1 M NaCl/5 mM MgCl₂]. Fractions containing Mm-cpn were precipitated with ammonium sulphate and resuspended in buffer C [25 mM Mops/NaOH (pH 7.2)/75 mM NaCl/5 mM MgCl₂] for gel-sizing chromatography on a Superdex S300 26/60 HiLoad column (Pharmacia). Resulting fractions were applied to a CBT-10 hydroxyapatite column (Bio-Rad Laboratories, Hercules, CA, U.S.A.) pre-equilibrated with 10 mM sodium phosphate buffer, pH 6.8. Elution from this column was achieved with a linear phosphate gradient from 10–500 mM. Mm-cpn protein was precipitated with ammonium sulphate, resuspended in buffer A, desalted on a Sephadex G75 26/10 column (Pharmacia) into buffer D [30 mM Tris/HCl (pH 7.5)/100 mM KCl/5 mM MgCl₂] supplemented with 10 % (v/v) glycerol, and stored at –80 °C.

A construct of the entire Mm-cpn apical domain, comprising residues Lys²¹⁰–His³⁶¹, was generated by PCR and subcloned into a pET30b vector. The vector was introduced into *E. coli* BL21(DE3) cells for expression as a C-terminally His₆-tagged fusion. The protein was purified under non-denaturing conditions by immobilized metal-affinity chromatography, using Ni²⁺-nitrilotriacetate ('Ni-NTA') Superflow resin (Qiagen, Chatsworth, CA, U.S.A.) according to the manufacturer's instructions, and sizing chromatography on a Superdex S300 column as described above.

ATPase activity of Mm-cpn

ATPase activity was determined by measuring liberated P_i in a Malachite Green assay as described in [18,19]. To determine the temperature-dependence of Mm-cpn ATPase activity, Mm-cpn

(215 nM final oligomer) was added to buffer D pre-equilibrated at various temperatures, as indicated in the Figure legends.

Rhodanese reactivation

Bovine rhodanese (60 μ M) was denatured in 6 M guanidinium chloride (GdmCl)/5 mM dithiothreitol (DTT) for 1 h at room temperature, and then diluted 150-fold into buffer D (supplemented with 2 mM DTT) at 37 °C, in the absence or presence of Mm-cpn or other chaperonins, as indicated in the Figure legends. After 15 min, samples were centrifuged to pellet aggregates and supplemented with 10 mM sodium thiosulphate. Reactivation was initiated by the addition of ATP or other indicated nucleotides (2 mM). For time-course analysis, reactivation was stopped at the indicated times with 10 mM EDTA. Rhodanese activity that was regained was measured as described previously [4,16].

Other techniques

GroEL and GroES were purified as described previously [4,16]. Thermally induced aggregation of pig citrate synthase was monitored by observing increased attenuation at 320 nM upon dilution of citrate synthase (1 μ M final monomer) into buffer D at 46 °C, in the absence or presence of various proteins, as indicated in the Figure legends. Electron-microscopy experiments were performed on a Philips 300 transmission electron microscope with an applied voltage of 60 kV. Mm-cpn samples were cross-linked with 0.1 % (v/v) glutaraldehyde for 60 s and desalted to remove excess fixative. Samples were then applied to Formvar and carbon-coated copper grids, and negatively stained with 2 % (w/v) uranyl acetate. Non-denaturing PAGE (3–10 % polyacrylamide gels) was performed as described previously [4].

RESULTS AND DISCUSSION

Cloning, recombinant expression, and purification of Mm-cpn

Desirable properties of a biochemical model system for type II chaperonins include a homo-oligomeric complex structure to facilitate recombinant expression and mutagenesis studies, and full functionality at room temperature. To establish such a system, we set out to clone chaperonin genes from mesophilic methanogens, based on the rationale that, although most known archaeal chaperonins form hetero-oligomeric complexes, the genomes of some (hyper)thermophilic methanococci contain only a single chaperonin gene [13,20]. The chaperonin described herein from *M. maripaludis* (Mm-cpn) fulfils all the desired criteria. After cloning the chaperonin gene from genomic DNA by PCR-related techniques, we confirmed that it was present as a single copy by Southern blotting and sequence analysis of degenerate PCR reactions (results not shown). The 543-amino-acid sequence of Mm-cpn (Figure 1) shows 84 %, 74 % and 33 % identity with the chaperonins from *M. thermolithotrophicus*, *M. jannaschii* and *Saccharomyces cerevisiae* CCT α respectively. Several features common to chaperonins are present in the Mm-cpn sequence, including the GDGTTT motif involved in ATP-binding, and the Gly-Met repeats at the C-terminus. Nucleotide sequences immediately upstream and downstream of the coding region (Figure 1) reveal several conserved elements that are probably important for the expression of the Mm-cpn gene *in vivo* [21–23]. The gene coding for Mm-cpn was introduced into *E. coli* BL21(DE3) cells for heterologous expression. During the purification process, anion exchange on a Mono Q column

Figure 1 Sequence of the chaperonin from *M. maripaludis*

Amino acid sequence of Mm-cpn (M.m.) is aligned with chaperonin sequences from *M. thermolithotrophicus* (M.t.; SwissProt accession no. Q93624), *M. jannaschii* (M.j.; accession no. Q58405), and CCT α from *S. cerevisiae* (S.c.; accession no. P12612). The GDGTTT motif and the Gly-Met repeats are identified by boxes. Asterisks denote residues of the helical protrusion. The genomic DNA sequence found immediately upstream of the start codon (lowercase 'atg') and immediately downstream of the stop codon (lowercase 'taa') in the Mm-cpn gene is indicated below the alignment. The horizontal lines denote putative transcriptional and translational cis-acting elements, including TATA box (tata), transcription-factor-B-binding site (ttf), ribosome-binding site (rbs) and T-rich transcriptional terminator (term).

was found to be a critical step in separating Mm-cpn from endogenous *E. coli* GroEL to ensure that any chaperone activity that was observed subsequently was not due to contaminating bacterial chaperonin. Mm-cpn is expected to exhibit the hallmark chaperonin double-ring structure. Consistent with this, purified Mm-cpn eluted on a Superdex S300 gel-filtration column as a 900 kDa complex (results not shown), migrated on native PAGE as a single high-molecular-mass species, and exhibited the typical ring-like structure under the electron microscope (Figure 2).

ATPase activity and stability of Mm-cpn as a function of temperature

Both the binding and hydrolysis of nucleotide are essential in modulating the function of a chaperonin during a typical reaction cycle [16,24]. The Mm-cpn oligomer hydrolyses ATP at a rate (mean $\pm$ S.E.M.) of 59.4 ± 2.1 ATP $\cdot$ min $^{-1}$ at 37 $^{\circ}$ C. Adhering to our goal of establishing a functional chaperonin system active at ambient temperatures, we determined the temperature-dependence of Mm-cpn protein stability and ATPase activity. ATPase activity reached a maximum at 50 $^{\circ}$ C, and declined sharply with further increases in temperature (Figure 2b). In contrast, the thermophilic archaeal chaperonins studied to date often have very little ATPase activity at ambient temperatures, and some display optimal activities only at temperatures in excess of 70 $^{\circ}$ C [10,12]. The stability of the complex correlated with ATPase activity. When Mm-cpn was incubated for 30 min at various temperatures and samples were spun down to pellet any

aggregates formed, analysis of the supernatants by native PAGE (Figure 2c) showed decreased amounts of soluble Mm-cpn upon incubation at 60 $^{\circ}$ C, with a complete loss of soluble Mm-cpn at 70 $^{\circ}$ C.

Chaperone activity of Mm-cpn

A defining characteristic of molecular chaperones is their ability to bind non-native proteins and prevent their aggregation in solution. As an initial test substrate for our studies, we chose pig citrate synthase, a homodimer of 48 kDa subunits. Citrate synthase has been shown to aggregate rapidly and irreversibly at temperatures above 43 $^{\circ}$ C [11]. Indeed, incubation of citrate synthase at 46 $^{\circ}$ C resulted in a rapid rise in attenuation at 320 nm, due to light scattering caused by the formation of aggregates (Figure 3). However, a 2-fold molar excess of Mm-cpn over citrate synthase (oligomer measured against subunits) was sufficient to completely suppress aggregation. The effect was specific to Mm-cpn, since a 5-fold excess of BSA did not suppress aggregation. Addition of ATP did not result in either a resumption of aggregation (Figure 3) or folding (results not shown), suggesting that Mm-cpn could bind, but not effectively release, thermally denatured citrate synthase in a folding-competent form.

To determine whether Mm-cpn possesses full chaperonin activity, we tested its ability to refold denatured bovine rhodanese, a monomer of 33 kDa that has been used widely as a model substrate in mechanistic studies of GroEL [4,16]. GdmCl-denatured rhodanese does not refold spontaneously

Figure 2 Mm-cpn ATPase activity and complex stability

(A) Transmission electron micrograph of purified Mm-cpn (1 μ M) fixed with 0.1% (v/v) glutaraldehyde, and negatively stained with 2% (w/v) uranyl acetate. Scale bar, 25 nm. (B) Mm-cpn (215 nM oligomer) was added to buffer D pre-equilibrated at the indicated temperatures (30–70 °C). ATPase activity was initiated by the addition of 2 mM ATP. (C) Mm-cpn (215 nM oligomer) was added to buffer D, pre-equilibrated at the indicated temperatures (30–70 °C), and incubated for 30 min. After centrifugation to remove aggregates, samples were analysed by non-denaturing PAGE on a 3–10% gel.

Figure 3 Chaperone activity of Mm-cpn

Citrate synthase (1 μ M monomer) was added to buffer D at 46 °C in the absence or presence of a molar excess of various proteins, as described below. Where indicated, ATP was added to a final concentration of 2 mM. Protein aggregation was determined by measuring increase in attenuance (D) at 320 nm. Grey diamonds, citrate synthase alone; black circles, BSA: citrate synthase at a ratio of 5:1; white squares, Mm-apical domain: citrate synthase at a ratio of 32:1; black squares, Mm-cpn: citrate synthase at a ratio of 2:1.

when diluted into buffer, but misfolds into aggregation-prone conformations. It requires the assistance of the complete GroEL/GroES system for folding in the chaperonin cavity. When we diluted rhodanese into buffer containing Mm-cpn present at a 1.5-fold molar excess, we consistently observed recovery of up to 70% of the enzyme activity (Figure 4). Recovery was nucleotide-dependent (Figure 4a). The half-life ($t_{1/2}$) of re-activation with Mm-cpn was 10 min compared with a

Figure 4 Nucleotide-dependent folding of rhodanese

Denatured rhodanese (60 μ M) was diluted 150-fold into buffer D supplemented with 2 mM DTT at 37 °C and containing a molar excess of various proteins, as described below. (A) Folding was initiated with 2 mM ATP. At the indicated time points, aliquots were removed and assayed for rhodanese activity. White circles, GroEL: rhodanese at a ratio of 1.5:1 and GroES: GroEL at a ratio of 4:1; grey squares, Mm-cpn: rhodanese at a ratio of 1.5:1; white squares, Mm-cpn: rhodanese at a ratio of 1.5:1 (no ATP added to initiate refolding); black circles, Mm-apical domain: rhodanese at a ratio of 32:1. (B) Folding was initiated with 2 mM of the indicated nucleotide. After 1 h, samples were assayed for rhodanese activity. 'spont' denotes activity of rhodanese diluted into buffer alone. In the remaining samples, Mm-cpn: rhodanese was present as a ratio of 1.5:1. AMP-PNP, p[NH]ppA.

$t_{1/2}$ of < 5 min with GroEL/GroES under equivalent experimental conditions (Figure 4a).

Substrate binding resides in the apical domain of chaperonin subunits. Previously, GroEL apical domain fragments, termed 'mini-chaperones', have been shown to possess limited chaperone activity *in vitro* [25]. We tested whether an individual Mm-cpn apical domain (Mm-apical) could likewise reversibly bind unfolded polypeptide. However, even a 32-fold molar excess of Mm-apical fragments had no effect on citrate synthase aggregation (Figure 3). Mm-cpn, BSA and Mm-apical were stable under the experimental conditions at 46 °C, and did not aggregate themselves (results not shown). Furthermore, Mm-apical could neither promote the refolding of rhodanese (Figure 4a) nor suppress its aggregation upon dilution from denaturant (results not shown). High-affinity binding of rhodanese, and presumably other substrates, to Mm-cpn might only occur in the context of an intact ring, which would provide multiple apical domain surfaces for efficient substrate interaction, as has been shown to be the case for GroEL [26].

Finally, we examined which nucleotide-bound state of Mm-cpn is active in refolding rhodanese, and whether or not ATP hydrolysis is necessary (Figure 4b). The folding of GdmCl-denatured rhodanese by GroEL is strictly dependent on the presence of GroES and nucleotide [4,16]. However, nucleotide hydrolysis by GroEL is not strictly required in this context, because folding can be effected by the non-hydrolysable analogue

p[NH]ppA, albeit with lower yield, and also by ADP [27]. In the case of Mm-cpn, we observed recovery of up to 70 % of rhodanese enzymic activity with ATP. We found that ADP could not support rhodanese folding, whereas the non-hydrolysable ATP analogue p[NH]ppA resulted in recovery of approx. 40 % of enzymic activity (Figure 4b). The ability of Mm-cpn to refold rhodanese in the presence of p[NH]ppA shows that binding of ATP is sufficient to enable folding of the substrate protein under these experimental conditions, and that hydrolysis of ATP is not essentially required. Small-angle neutron-scattering studies have suggested that p[NH]ppA can effect conformational changes in the chaperonin from *Thermoplasma acidophilum*, which were interpreted as an open-to-closed transition upon binding of ATP [28]. Cryo-electron-microscopic reconstruction of CCT in the presence of p[NH]ppA likewise revealed the chaperonin to be in a closed conformation [29]. Also, p[NH]ppA was recently demonstrated to promote folding of green fluorescent protein in a thermophilic archaeal chaperonin system from *Thermococcus* strain KS-1 [5]. That study, however, employed recombinantly produced homo-oligomeric chaperonin complexes, which, in contrast with Mm-cpn, exist as hetero-oligomers *in vivo* [10]. Mutagenesis of the Mm-cpn ATP-binding site and the helical protrusion, which is supposed to reversibly occlude the ring interior, should clarify further the role of nucleotide in the opening and closing of the chaperonin cavity.

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