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An exceptionally stable Group II chaperonin from the hyperthermophile *Pyrococcus furiosus*

Haibin Luo, Pongpan Laksanalamai, Frank T. Robb *

Center of Marine Biotechnology, University of Maryland Institutes, 701 East Pratt Street, Baltimore, MD 21202, USA

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ABSTRACT

The hyperthermophilic archaeon *Pyrococcus furiosus* (Pf) grows optimally at 100 °C and encodes single genes for the Group II chaperonin (Cpn), Pf Cpn and α-crystallin homolog, the small Heat shock protein (sHsp). Recombinant Pf Cpn is exceptionally thermostable and remained active in high ionic strength, and up to 3 M guanidine hydrochloride (Gdn-HCl). Pf Cpn bound specifically to denatured lysozyme and ATP addition resulted in protection of lysozyme from aggregation and inactivation at 100 °C. While complexed to heat inactivated lysozyme, Pf Cpn showed enhanced thermostability and ATPase activity, and increased the optimal temperature for ATPase activity from 90 to 100 °C. Protein substrate binding also stabilized the 16-mer oligomer of Pf Cpn in 3 M Gdn-HCl and activated ATPase hydrolysis in 3–5 M Gdn-HCl. In addition, Pf Cpn recognized and refolded the non-native lysozyme released from Pf sHsp, consistent with the inferred functions of these chaperones as the primary protein folding pathway during cellular heat shock.

Introduction

The inventory of molecular chaperones constitutes the cellular system that assists folding and assembly of newly synthesized proteins, translocation of unfolded proteins across membranes, as well as refolding and degradation of misfolded and aggregated proteins [1]. Chaperonins (Cpns)[1] are large protein folding complexes that are able to accommodate a wide range of unfolded, misfolded or partially folded proteins in order to promote correct folding in an ATP dependent manner [2]. They are divided into Group I chaperonins, represented by GroEL found in all Bacteria, mitochondria and chloroplasts, and Group II chaperonins, occurring in eukaryotes and archaea [3,4]. Group I chaperonins are composed of 14 identical subunits arranged in two heptameric rings and requires a co-chaperone, GroES, to facilitate protein folding. Group II chaperonins form torodal double rings with an eight or ninefold rotary symmetry and functions without a co-chaperone [5]. Group I and Group II chaperonins share similar quaternary structures consisting of a double toroid cylinder assembled into two rings stacked back to back. This arrangement generates two internal cavities that allow unfolded proteins to fold correctly in a confined environment [2,6]. The archaeal Group II chaperonins are double-ring complexes. As befits a class of proteins with vital functions, there are frequently multiple paralogs encoding nonidentical but related products that can be assembled into a wide variety of functional chaperone complexes with either identical or nonidentical subunits. At least two chaperonin homologs are present in most archaeal genomes published to date. *Pyrococcus furiosus*, in common with some thermophilic methanogens (e.g. *Methanopyrus kandleri, Methanococcus jannaschii* (*Methanocaldococcus jannaschii*), and *Methanococcus thermolithrotrophicus*) have chaperonins with identical subunits. Some archaea have three or more chaperonin subunits, for example *Sulfolobus* spp. contain three different subunits and there are five chaperonin subunits (Hsp60-1, -2, -3, -4, and -5) in *Methanosarcina acetivorans* [3].

Although definitive mechanistic and structural studies have been completed on the Group I GroEL/GroES chaperonins from *E. coli*, information on the mechanism of protein folding by archaeal Group II chaperonins and the mechanisms of their cooperation with other members of the protein folding pathway is incomplete or lacking. *In vitro* protein folding activity has only been investigated in a few archaeal Group II chaperonins [6–8]. *Pyrococcus furiosus* (Pf), a thoroughly characterized hyperthermophilic archaeon, grows optimally at 100 °C. However, the stability and ATPase activity of Pf Cpn at or near 100 °C has not been characterized well.

In a previous study, we established that Pf Cpn (or Hsp60) is able to protect *Taq* DNA polymerase at high temperature in a cooperative reaction with either prefoldin or small Heat shock protein (sHsp) co-chaperones [9]. In contrast to other chaperone families, small Heat shock proteins (sHsps) bind many non-native proteins per oligomeric complex, thus representing efficient "holdase" chaperones in terms of the substrate binding [10]. Since sHsps

* Corresponding author. Fax: +1 410 234 8896.
E-mail address: robb@umbi.umd.edu (F.T. Robb).

[1] *Abbreviations used:* Pf, *Pyrococcus furiosus*; Hsp, Heat shock protein; Cpn, chaperonin; sHsp, small Heat shock protein.

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are known not to carry out active protein refolding, and do not carry out ATP hydrolysis, the transfer of unfolded proteins from sHsps to Cpns represents a potential pathway salvage mechanism for recycling proteins during episodes of cellular stress [11,12]. In Group I chaperonins, the release of substrate proteins from the sHsp complex is achieved in cooperation with Hsp70 (DnaK) in an ATP-dependent reaction [10]. Group I and Group II chaperonins, prefoldins and sHsps play major roles in the molecular chaperone systems of archaea, and occur as multiple duplicated or paralogous gene copies in most of the archaeal genomes studied to date [13].

We have previously proposed that the reduced genome sizes of hyperthermophiles constrain the copy number of many gene families, including chaperones, resulting in minimal complexity cellular protein folding systems consisting of reduced numbers of versatile chaperones [13]. The genome of *Pyrococcus furiosus*, in common with several other hyperthermophilic Archaea, encodes such a minimal protein folding system consisting of single copies of Cpn and sHsp, and single copy *pfd* α and *pfd* β genes encoding an $\alpha_2\beta_4$ prefoldin complex [14]. DnaK/J-GrpE chaperones, present and apparently essential in all eukarya, are absent as are the bacterial-type Group I chaperonins that are found in many moderately thermophilic and halophilic Archaea. Cooperative protein folding between recombinant Cpn and prefoldin from *Pyrococcus horikoshii* and *Thermococcus* KS-1 have been studied [7,8,15–18]. However, the specific mechanism of exchange of misfolded proteins from sHsp to Pf Cpn was not revealed.

In the present study, we determine the intrinsic stability of Pf Cpn and study the cooperative substrate exchange from Pf sHsp to Pf Cpn, moving towards understanding of the molecular mechanism of a major heat-shock inducible protein folding pathway in the Archaea.

Materials and methods

Chemicals, enzymes and reagents

Escherichia coli strains used in this study were DH5α for the initial cloning of the pET expression plasmid and BL21(DE3) for the recombinant protein expression. Restriction enzymes, polymerase, and ligase were purchased from New England Biolabs (Beverley, MA). All other chemicals were of analytical grade from Sigma–Aldrich (St. Louis, MO). All solutions were made up in ultra-pure water. NativeMark™ unstained molecular weight protein standard was from InVitrogen (Carlsbad, CA).

Cloning, expression and purification of Pf Cpn and sHsp

The Pf Cpn gene was amplified from *Pyrococcus furiosus* genomic DNA [14] using primers containing NcoI and BamHI: sense, 5′-CCATGGCCCAGTTAGCAGGC-3′; antisense, 5′-GGATCCTCAGTCTAGATCACTGCTGAAGTCCTCGC-3′. The gene fragment was inserted into a pCR-Blunt vector (InVitrogen, Carlsbad, CA).The construct was digested by NcoI and BamHI and subsequently inserted into pET-11-d expression vector (Novagen, Madison, WI). The construct was identified by PCR with the corresponding primers and the fragments were checked by digestion with the same restriction enzymes. The nucleotide sequence of the construct was confirmed by DNA sequencing. The recombinant plasmid was transformed into *E. coli* strain BL21(DE3) and inoculated into 10 ml of LB medium plus 100 μg/ml ampicillin at 37 °C, this 10 ml culture was transferred into 1 L of LB medium with the same antibiotics 10 h later. Growth at 37 °C was monitored by measuring OD_{600} and IPTG was added at 0.8 OD to a final concentration of 0.5 mM. Induction was allowed to proceed at 30 °C for 4 h. The cells were harvested by centrifugation at 6000*g* for 10 min and stored at −80 °C. Harvested cells were suspended in extraction buffer (25 mM Hepes–KOH, pH 7.2, 100 mM KCl) lyzed by French Press and centrifuged at 25,000*g* for 30 min. The supernatant extract was heated to 80 °C for 30 min and rapidly cooled down on ice. The aggregated proteins were removed by centrifugation at 25,000*g* for 30 min. The supernatants were then further purified to homogeneity by anion exchange (Q matrix). The protein concentration was determined by the Bradford assay.

Pf sHsp was cloned, expressed and purified as previously described [19].

ATP hydrolysis activity

Nucleotide hydrolysis activity was determined by Pi release using a standard assay [8,20,21]. The standard reaction mixture contained 25 mM Hepes–KOH, pH 7.2, 300 mM KCl, 10 mM $MgCl_2$, 0.02 mg/ml Pf Cpn, and 200 μM ATP in a final volume of 90 μl. ATPase activity of Pf Cpn was assayed at 90 °C for 15 min unless noted otherwise. The reaction was stopped with 2% perchloric acid and the liberated Pi was determined by the Malachite Green assay at 630 nm [20]. The buffers used for determining pH dependence of Pf Cpn are listed as following: pH 2.5–3.5, Glycine–KOH; pH 4.0–6.0, Glutamate–KOH; pH 6.5, Pipes–HCl; pH 7.0–8.5, Hepes–KOH; pH 9.0–10.0, Na_2CO_3–$NaHCO_3$; pH 11, Na_2CO_3–NaOH. Light mineral oil was used to prevent evaporation when the assay temperature was higher than 100 °C.

Surface plasmon resonance binding analysis

The surface plasmon resonance experiments were carried out on a BIAcore T100 biosensor system (GE Healthcare BIAcore AB, Uppsala, Sweden). Pf Cpn and sHsp were covalently coupled to the experimental flow cell on the CM5 sensory chip via the standard amine coupling procedure. In rapid succession, 50 μg/ml protein in 10 mM sodium acetate (pH 4.5) was injected on the sensor chip surface previously activated by *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N*-(dimethylaminopropyl)carbodiimide (EDC). Ethanolamine was then injected to quench the unreacted *N*-hydroxysuccinimide groups. A control flow cell was activated and blocked without coupling protein for use as the reference. The system running buffer used HBS buffer (10 mM Hepes, pH 7.4, 150 mM NaCl). Analytes were prepared in the same buffer and injected at a flow rate of 30 μl/min. After one test cycle, the assay flow cell was regenerated by washing bound analytes from immobilized ligand with buffer and 50 mM NaOH. The bound chaperones were unaffected by NaOH treatment. All binding experiments were carried out at 42 °C.

Heat-induced inactivation and aggregation assays of lysozyme

Mixtures of lysozyme with Pf Cpn or ATP were incubated at 100 °C for the indicated time and subsequently assayed for lysozyme activity or turbidity. Lysozyme activity determination was carried out according to the published method [22,23] with modification as follow. Samples were removed from heat at the indicated time points and mixed with *Micrococcus lysodeikticus* cells (a generous gift from Zylacta Corporation) to a final concentration of 0.30 mg/ml in a total volumne of 50 μl, 0.1 M potassium phosphate (pH 6.2). The decrease in absorbance at 450 nm was recorded. The relative activity of lysozyme was determined as a percentage ratio in comparison to a control of the same sample after 100 °C, heat treatment. Protein aggregation was monitored as turbidity at 450 nm; the absorbance of lysozyme heated at 100 °C for 60 min was considered as 100% aggregation.