Purification and structural characterization of the thermosome from the hyperthermophilic archaeum *Methanopyrus kandleri*

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Abstract From *Methanopyrus kandleri*, the most thermophilic methanogen known so far, we have purified to homogeneity a protein complex of high molecular mass. Image analysis of transmission electron micrographs revealed a barrel-shaped particle composed of two rings with 8-fold symmetry. Only one type of subunit could be detected. The corresponding gene has been cloned and sequenced. The deduced amino acid sequence shows high homology with the members of group II chaperonins. The structure of the projection and the sequence homology suggest that this particle is the first thermosome isolated from a methanogen.

Key words: Archaea; Chaperonin; Thermosome; Methanopyrus

1. Introduction

Chaperonins are multisubunit ATPases implicated in the correct folding of nascent or non-native proteins [1,2]. The toroidal particles are composed of two rings, stacked back to back. Each ring is built by one or several types of protein subunits with molecular masses of about 60 kDa. The chaperonins can be divided into two groups [3]. Group I chaperonins (GroEL family) are found in bacteria, chloroplasts and mitochondria. They are composed of one or two different subunits, forming rings with sevenfold radial symmetry. Members of the group II chaperonins (TCP1/thermosome family), found in the eukaryotic cytosol and in Archaea [4], also form toroidal structures, but with variations regarding the number of different subunits and the rotational symmetry of the rings. T complex polypeptide 1 (TCP1) is part of a heteromeric ring complex (TRiC [5], also named CCT [6]), localized in the eukaryotic cytosol. This complex contains 7 to 9 different polypeptides (M_r 50000–68000) [7,8]. It has been shown that CCT is involved in the folding of actin, tubulin and fire fly luciferase in vitro [5,9,10] and that newly synthesized actin and tubulin monomers are bound by CCT in vivo [11].

The first chaperonins characterized in Archaea were thermophilic factor 55 (TF55) from *Sulfolobus shibatae* [12] and the thermosome from *Pyrodictium occultum* [13,14]. TF55 is the major heat shock protein in *S. shibatae* [15]. Its ninefold symmetric rings are composed of subunits with an apparent molecular mass of about 55 kDa. Recently a second subunit in this organism (TF56, GenBank accession no. P46219) has been identified. The hitherto available data are not sufficient to judge whether homo- or hetero-oligomeric complexes are formed in vivo. TF55 isolated from *S. solfataricus* [16] consists of two different polypeptides (M_r 58000 and 61000). From reconstitution experiments the authors concluded, that both subunits are contained within the same particle.

The thermosome of *Pyrodictium occultum* [17] comprises two different subunits (M_r 56 000 and 59 000) in a 1:1 ratio which form rings with pseudo 8-fold symmetry. The complex is a very thermostable ATPase and accumulates during growth at the upper temperature limit (110°C) [13,14]. Using polyclonal antibodies, raised against the *Pyrodictium* thermosome, the existence of immunologically related proteins in a wide variety of thermophilic and hyperthermophilic Archaea has been demonstrated [13]. The strongest signals have been obtained with *Sulfolobus acidocaldarius* and *Thermoplasma acidophilum*. Recently, the thermosome from *T. acidophilum* has been isolated and characterized [10,18,19]. With the exception of *Methanothermus fervidus*, no or only weak signals were obtained with mesophilic, thermophilic and hyperthermophilic methanogens and halophilic Archaea. Nevertheless the key functions of chaperonins in protein folding suggest that related proteins exist in these organisms.

In this report we describe the purification and molecular cloning of a high molecular weight protein complex from the hyperthermophilic *Methanopyrus kandleri* [20,21]. The projection structure of the particle and sequence analysis of the subunit clearly show, that we have isolated the first thermosome from a methanogen.

2. Materials and methods

2.1. Cell culture

Methanopyrus kandleri was grown in a 300 l titanium coated fermenter on H_2 and CO_2 at 98°C in BS medium [21]. Cells were harvested at the end of exponential growth phase and stored at −80°C.

2.2. Purification of Methanopyrus thermosome

The protein complex was purified from 13 g (wet weight) *Methanopyrus kandleri* cells under the exclusion of oxygen in an anaerobic chamber (Coy Manufacturing Company, Ann Arbor, MI).

Thawed cells were suspended in 7 ml TM buffer (50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 0.1 M NaCl, 20% glycerol) and lysed by two passes through a French pressure cell at 20 000 psi. Cell debris were removed by ultracentrifugation (1 h, 40,000 rpm, 4°C, 60 Ti rotor). The clear supernatant was loaded onto a Q Sepharose column (2 cm × 25 cm) equilibrated in TM buffer. Bound proteins were eluted with a linear gradient of NaCl (100–1000 mM in TM buffer). Fractions from 380 to 570 mM NaCl were pooled, diluted with two volumes TM buffer and applied to a Heparin-Sepharose-column (1 cm × 10 cm) equilibrated in TM buffer. After step elution (1000 mM NaCl) thermosome-containing fractions were pooled, desalted and concentrated by chromatography on Mono Q HR 5/5 (Pharmacia, Freiburg, Germany) with subsequent

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step elution (1000 mM NaCl). The Mono Q eluate was size fractionated on a HiLoad 16/60 Superdex 200 column (Pharmacia, Freiburg, Germany) in TM buffer containing 500 mM NaCl.

Thermosome containing fractions eluting in the void volume were pooled, diluted twofold with TM buffer and applied to a Mono Q HR 5/5 column equilibrated in TM buffer supplemented with 200 mM NaCl. Bound proteins were eluted with a linear NaCl gradient (200 mM–1000 mM in TM buffer; 40 ml).

From 13 g cells 9–11 mg pure thermosome were obtained.

2.3. Transmission electron microscopy and image analysis

Purified complexes were negatively stained with 2% (w/v) ammonium molybdate. Micrographs were recorded at a magnification of 33,600 under low dose conditions using a Phillips CM12. Non overlapping areas were digitized as arrays of 2048 × 2048 pixels with an Eikonix 1412 camera system at a pixel size of 20 μm, corresponding to 0.446 nm at the specimen level.

The alignment and averaging of side-views was performed with the help of an interactively chosen reference in an iterative way. For the analysis of the particles in end-on orientation a method based on eigenvector, eigenvalue data analysis was used as described elsewhere [22].

2.4. Determination of peptide sequences

200 μg thermosome were digested with 1 μg trypsin in 200 mM $(NH_4)_2CO_3$ pH 8.8 in a total reaction volume of 100 μl. Resulting peptide fragments were isolated by reversed phase chromatography and N-terminally sequenced by automated Edman degradation.

2.5. PCR-amplification, cloning, and sequencing

From internal amino acid sequences 2 primers were designed:

MKthsF1: 5′-CAG GAG GAC GAG $GT^T/_G$ $GG^T/_G$ GAC GAC-3′ (forward)

MKthsR1: 5′-GCG $TC^T/_G$ GCG $AA^T/_G$ GCC $TC^T/_G$ $AC^T/_G$ GC-3′ (reverse)

50 ng *M. kandleri* chromosomal DNA and 10 pMol of each primer were used in a 50 μl reaction mixture. The reaction was overlaid with 20 μl Chill-out wax (Biozym, Hess. Oldendorf, Germany) to prevent evaporation, heated to 94°C for 1.5 minutes, and then subjected to 30 cycles of amplification (90″ 94°C, 60″ 55°C, 90″ 72°C) followed by a polymerization step at 72°C for 15 minutes. Subsequently, the tubes were chilled on ice, the liquid phase was removed and aliquots of the reaction products were analyzed on 1% agarose gels. Gel-purified PCR products (~1000 bp) were DIG-labeled and used for screening a genomic λ-library of *Methanopyrus kandleri*. From 5 positive clones a 3.6 kbp *Sac*I-fragment was subcloned into pUC18. For sequencing, overlapping deletion clones from both strands were generated using Exonuclease III (Nested deletion kit, Pharmacia, Freiburg, Germany). Sequence alignment and database searches were performed using the UWGCG software package (University of Wisconsin Genetics Computer Group) [23].

2.6. SDS-PAGE and protein determination

SDS-PAGE was performed as described [24]. For high resolution SDS-PAGE a Tricine-based gel system was used [25].

Protein concentrations were determined by the method of Bradford et al. [26].

Table 1
Pairwise comparison of the *Methanopyrus* thermosome with group II chaperonins

	% Identity	% Similarity
S. shibatae TF55	554	762
S. shibatae TF56	541	745
P. spec.	693	854
T. acidophilum ThsA	591	776
T. acidophilum ThsB	579	751
human TCP1	383	617
E. coli GroEL	276	538

Homology scores as calculated with BESTFIT.

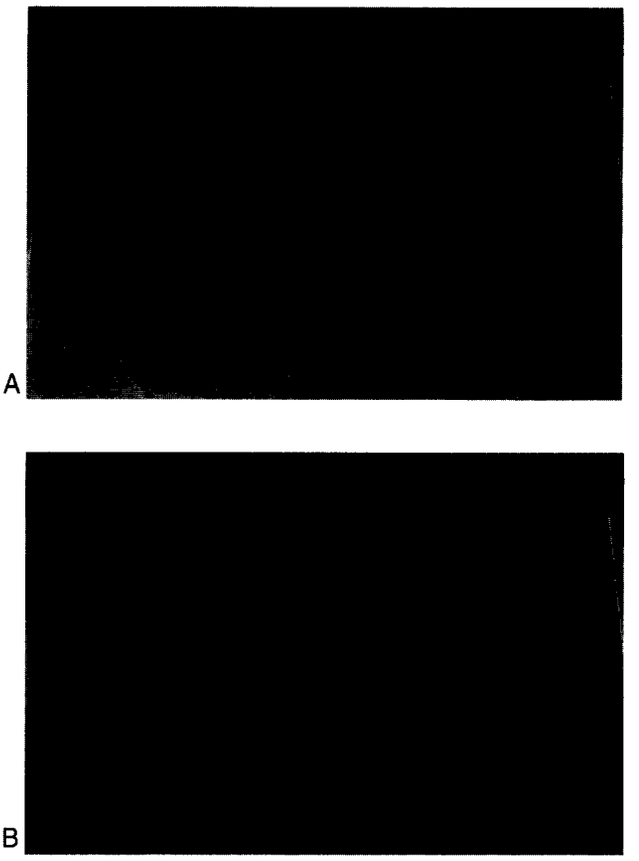

Fig. 1. SDS-PAGE. (A) Analysis of different purification steps of the DNA-dependent RNA polymerase from *Methanopyrus kandleri*. Lane 1, 8: molecular size standards (170 kDa; 116 kDa; 85 kDa; 55.5 kDa; 39 kDa; 26.6 kDa; 20 kDa; 14.3 kDa); lane 2, S-100; lane 3–6, active fractions from chromatography on Q-sepharose, heparin-sepharose, Mono Q, and Superdex 200; Lane 7, purified DNA-dependent RNA polymerase from *Methanococcus thermolithotrophicus*. (B) Analysis of the main peak fraction of the final Mono Q. Lanes 1,4, molecular size standard (see Fig. 1A); lane 2, proteins eluting at 470 mM NaCl; lane 3, proteins eluting at 600 mM NaCl.

2.7. ATPase assay

Samples containing 20 mM Tris-HCl pH 8.5, 10 mM $MgCl_2$, 100 mM KCl, 0.1 mM ATP, and 5 μCi [γ-^{32}P]ATP in a total reaction volume of 50 μl were equilibrated to assay temperature (90°C) for 5 min. The reaction was started by the addition of 2.5 μg purified thermosome. Enzymatic activities were stopped by rapid cooling to 0°C. ATP hydrolysis was examined by spotting aliquots (2 μl) on PEI cellulose plates (Merck, Darmstadt, Germany). P_i was separated from ATP by development in 0.8 M $(NH_4)_2SO_4$ and detected on Kodak X-AR film.

In an other set of experiments the assay conditions were varied as follows: Tris-HCl was substituted by HEPES/KOH pH 8.5; $MgCl_2$ and KCl concentrations were tested from 0–50 mM and 0–1 M, respectively. KCl was also replaced by NH_4Cl or KP_i and the pH was varied from 3–8.5 in 0.5 intervals (pH 3–6 in 50 mM citrate buffer).

Additionally, experiments were performed without labeled ATP, using a luciferin/luciferase system (ATP Bioluminescence CLS kit, Boehringer Mannheim, Germany) and a Lumac Biocounter for quantification of residual ATP [13].

3. Results and discussion

During the purification of the DNA-dependent RNA polymerase of *Methanopyrus kandleri* we recognized a polypeptide of 75 kDa (as revealed by SDS-PAGE) which is highly enriched

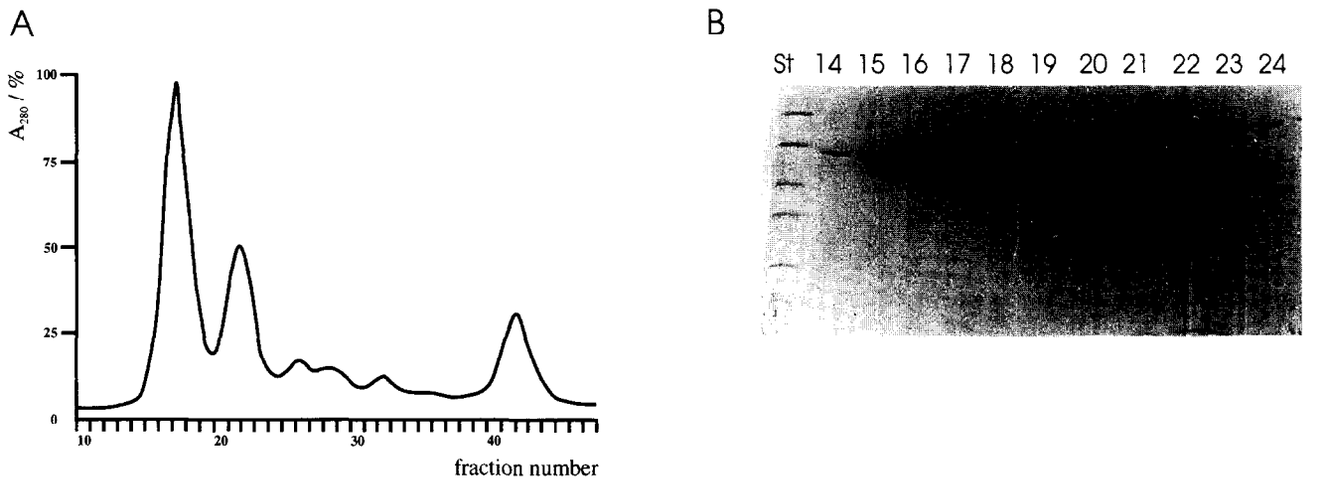

Fig. 2. Gel filtration chromatography. (A) Elution profile of the Superdex 200 column. (B) Column fractions were analyzed by SDS-PAGE. Numbers at the top correspond to the fractions numbers shown in A. St.: molecular size standards (116 kDa; 85 kDa; 55.5 kDa; 39 kDa; 26.6 kDa; 20 kDa).

by affinity chromatography on heparin-sepharose (Fig. 1, lane 4). This protein has been purified by chromatography of an ultracentrifuged crude extract (S-100) on Q-sepharose, heparin-sepharose, Superdex 200, and Mono Q HR5/5 (Fig. 1B, lane 2). The protein comigrates during the first two purification steps with the DNA-dependent RNA polymerase. It can be separated from the RNA polymerase by gel filtration on Superdex 200. A polypeptide with an apparent molecular weight of 75 kDa is the main component of the first peak (Fig. 2) indicating that the polypeptide is part of a high molecular weight complex. Purification to homogeneity could be achieved by chromatography on a Mono Q column and elution of bound proteins with a salt gradient (0.2 M–1.0 M NaCl in 40 column volumes). Under these conditions bound proteins were eluted in two separate peaks. The 75 kDa protein was released from the Mono Q with 470 mM NaCl (Fig. 1B, lane 2), smaller proteins eluted at 600 mM NaCl (Fig. 1B, lane 3).

The highly purified protein complex was examined by transmission electron microscopy. On micrographs, negatively stained particles appear with about equal numbers in two orientations (Fig. 3A). A ring-shaped view (end-on view) with eight segments surrounding a dark, stain filled center, and a rectan-

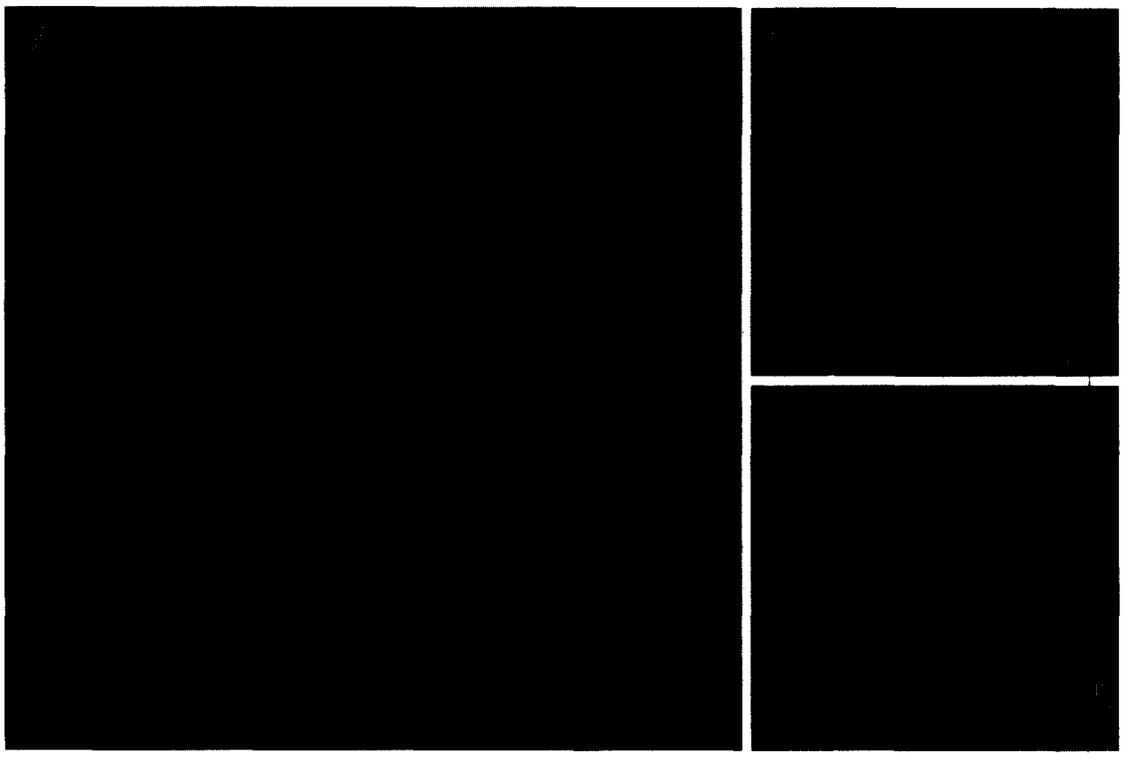

Fig. 3. (A) Transmission electron micrograph of the purified complex, negatively stained with ammonium molybdate, showing end-on (1) and side-on (2) views. Correlation averages with contour lines of 500 end-on (B) and 600 side-on (C) views of the same complex. (a, 4.3 nm; b, 14.5 nm; c, 13.6 nm)

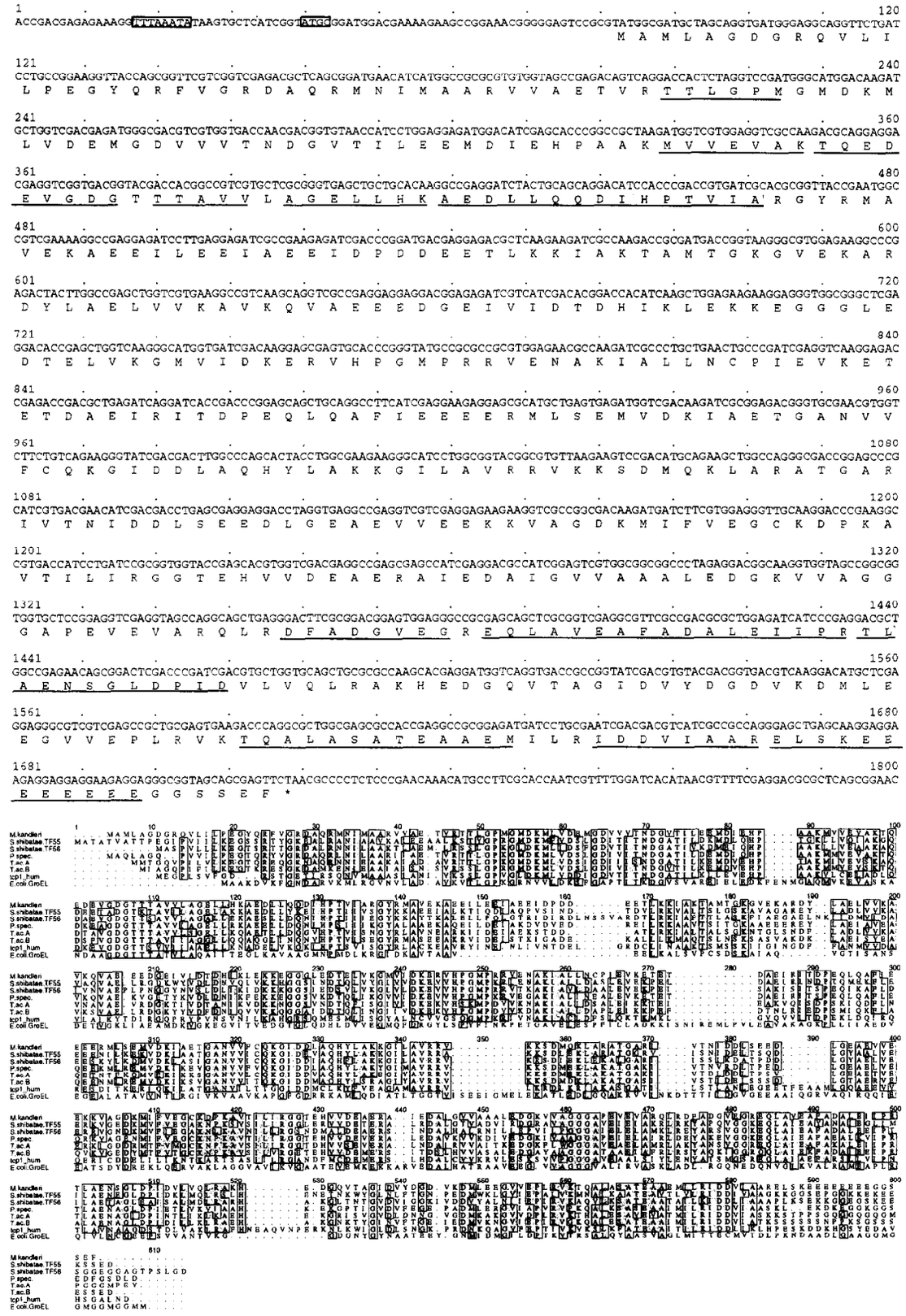

Fig. 4. (A) DNA fragment containing the *Methanopyrus* ths (thermosome subunit) gene. Sequences of tryptic peptides found in the deduced amino acid sequence are underlined. Peptides used for designing degenerate PCR primers are shadowed. A putative TATA-box and a transcription initiation element in front of the gene are boxed. The nucleotide sequence reported in this paper has been submitted to the EMBL data bank, accession number Z50745. **B:** Multiple sequence alignment of the *Methanopyrus* thermosome with known archaeal chaperonins, human TCP1, and bacterial GroEL. The alignment includes a heat-shock protein sequence from *Pyrococcus* strain KOD (D29672), TF55 and TF56 from *Sulfolobus shibatae* (P28488, P46219), the thermosome subunits from *Thermoplasma acidophilum* (Z46649 and Z46650), human TCP1 (P17987), and GroEL from *E. coli* (P06139).

gular view (side-on view) with two pairs of parallel striations. Image processing without symmetrization was applied to 600 side-on views and to 500 end-on views. The end-on view average clearly depicts a rotationally symmetric ring, 14.5 nm in diameter, with a large cavity, 4.3 nm in diameter (Fig. 3B). In the average of the side-on view, the striations are resolved into four strong centers of mass in the equatorial region of the complex and four weak centers in apical region. The connectivity between the equatorial and the apical region appears relatively weak (Fig. 3C). The particle clearly resembles the projection structure of other members of the group II chaperonins. But in contrast to the thermosomes from *P. occultum* [13,14], *T. acidophilum* [18] and the other members of this protein family, which are built from two or more different subunits, this complex appears to be a homo-oligomer composed of two 8-fold symmetric rings. Attempts to identify a second subunit in the complex by RP-HPLC, high resolution SDS-PAGE [25] or two-dimensional SDS-PAGE [27] have failed (data not shown).

Twelve tryptic peptide fragments of the *Methanopyrus* complex were isolated and N-terminally sequenced. With degenerate oligonucleotide primers derived from two peptide sequences a 1033 bp PCR product was amplified and cloned. This fragment was used to screen a λ-library constructed from *M. kandleri* chromosomal DNA. From five positive plaques a 3.6 kbp *Sac*I-fragment was subcloned into pUC18 and sequenced on both strands. The *Sac*I-fragment contains an open reading frame (Fig. 4A) which codes for an acidic polypeptide (pI = 4.27) of 545 amino acids with a calculated molecular weight of 59474 Da. Within this sequence, all 12 peptide sequences, derived from the purified protein, have been found. This clearly shows that the corresponding gene has been cloned and supports the homo-oligomeric nature of the purified complex. The relatively large difference between the sequence derived (59.5 kDa) and the apparent molecular weight on SDS polyacrylamide gels (75 kDa) may be due to the low isoelectric point (pI = 4.27) of the polypeptide. It has been reported that acidic proteins, probably due to restricted SDS-binding, may give too high molecular masses in SDS-PAGE [28].

The derived polypeptide sequence has high similarity to TF55 and TF56 from *S. shibatae* and the thermosome of *T. acidophilum* (Table 1). The *Methanopyrus* thermosome sequence can be aligned unambiguously over the entire length to the archaeal chaperonins and TCP-1 from eukaryotes (Fig. 4B). The alignment with bacterial GroEL shows only a weak similarity (27% identity, 54% comparative each).

Most closely related to the *Methanopyrus* chaperonin is a putative heat shock protein identified in the genome of a *Pyrococcus* species (strain KOD, Izawa et al., GenBank accession no. D29672). The two proteins are to 69.3% identical, whereas the chaperonin subunits isolated from *S. shibatae* and *T. acidophilum* are identical only to 54.1% and 59.8%, respectively. Unfortunately, the protein complex from this *Pyrococcus* species has not yet been purified and no data concerning the subunit composition are available.

A highly conserved, putative ATP-binding site (96-GDGTTT-101) [3,29] could be identified in the *Methanopyrus* thermosome sequence. However, we have failed so far to measure significant enzymatic ATP hydrolysis under several conditions tested (see section 2). Further biochemical and functional studies to investigate the catalytic properties of the *Methanopyrus* thermosome are in progress.

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