Thermostable chaperonin from *Clostridium thermocellum*

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Homologues of the chaperonins Cpn60 and Cpn10 have been purified from the Gram-positive cellulolytic thermophile *Clostridium thermocellum*. The Cpn60 protein was purified by ATP-affinity chromatography and the Cpn10 protein was purified by gel-filtration, ion-exchange and hydrophobic interaction chromatographies. The identities of the proteins were confirmed by N-terminal sequence analysis and antigenic cross-reactivity. The Cpn60 homologue is a weak, thermostable ATPase ($t_{1/2}$ at 70 °C more than 90 min) with optimum activity (k_{cat} 0.07 s⁻¹) between 60 °C and 70 °C. The ATPase activity of the authentic

Cpn60 was inhibited by *Escherichia coli* GroES. The catalytic properties of a recombinant *C. thermocellum* Cpn60 purified from a GST-Cpn60 fusion protein expressed in *E. coli* [Ciruela (1995) Ph.D. Thesis, University of Kent] were identical with those of the authentic *C. thermocellum* Cpn60. Gel-filtration studies show that at room temperature the Cpn60 migrates mainly as a heptamer. Electron microscopy confirms the presence of complexes showing 7-fold rotational symmetry and also reveals a small number of particles that seem to be tetradeecamers with a similar structure to *E. coli* GroEL complexes.

INTRODUCTION

Chaperonins are a family of ubiquitous and highly conserved proteins that function as molecular chaperones [1]. They comprise a large oligomer (14 identical subunits of 60 kDa each) known as Cpn60 (or GroEL in *Escherichia coli*) and a small oligomer (seven identical subunits of 10 kDa each) known as Cpn10 (or GroES in *E. coli*).

The chaperonins were originally recognized as factors required for bacteriophage assembly in *E. coli* [2]. They are now known to be involved in the folding of nascent polypeptides [3], the assembly of oligomeric protein complexes [4] and protein export from bacteria [5].

Cpn60/Cpn10 proteins homologous with *E. coli* GroEL/ES have been found in eubacteria, plastids and mitochondria [1]. These proteins are essential for cell viability and are produced during normal cell growth. Under stress conditions such as heat, toxic or solvent shock these proteins are transiently induced and expressed at higher levels [6], conferring enhanced resistance to stressful conditions. Many eubacterial chaperonins have been identified by cloning studies [7–9] and expressed in *E. coli*, but knowledge of the roles of chaperonins in bacterial physiology is based almost exclusively on studies of the *E. coli* GroEL/ES system. Given the established roles of chaperonins in protein folding, translocation and complex assembly, the characterization of these proteins in a wider range of bacteria might help to elucidate the general roles of chaperonins in bacterial physiology and to highlight novel functions.

Clostridium thermocellum is a Gram-positive, thermophilic anaerobe that produces an extracellular enzyme system capable of degrading crystalline cellulose to soluble sugars [10]. The cellulolytic enzymes form a large extracellular complex (the cellulosome) with a molecular mass of approx. 2.5 MDa [11,12]. At least 19 different genes have been cloned encoding cellulosomal enzymes (reviewed in [13–15]). One cellulosomal protein (S₁, also

known as CipA) has been shown to have specific binding sites for cellulose [16] and for cellulosomal enzymes [17]. CipA is thought to mediate the adhesion of bacterial cells to cellulose and to form a 'scaffold' around which the catalytic subunits assemble [18]. Currently little is known about the translocation of the cellulosomal components to the cell surface, or about the site and mode of assembly of the complex.

During our investigation of methods for enhancing the yield of cellulosomal proteins from *C. thermocellum*, we discovered that washing the cell pellet from a stationary-phase *C. thermocellum* culture resulted in the preferential release of predominantly two non-cellulolytic proteins. Sonication of the cell pellet is required for optimum release of these two proteins. In this paper we describe the identification of these proteins as homologues of the *E. coli* chaperonins GroEL and GroES, their purification and the characterization of the ATPase activity of the *C. thermocellum* Cpn60.

MATERIALS AND METHODS

Culture conditions for *Clostridium thermocellum*

C. thermocellum (N.C.I.M.B. no. 10682) was grown in CM3 medium [19]. Inoculum was grown in 10 ml sealed tubes under a nitrogen atmosphere at 60 °C with 0.25 % Sigmacell 20 (Sigma) as carbohydrate source. For protein extraction, 10 litre cultures were grown to stationary phase (4 days) in sealed flasks under the same culture conditions with 0.5 % cellobiose (Sigma) as the carbon source.

TEA buffer wash

The bacterial cells were harvested by centrifugation (3000 g, 10 min, 4 °C), resuspended in distilled water and vortex-mixed

Abbreviations used: PVDF, poly(vinylidene difluoride); TEA buffer, 100 mM triethanolamine adjusted to pH 6.9 with maleic acid.

‡ This work was initiated by Marek Romaniec shortly before his death in November 1992.

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briefly (15 s). The cells were pelleted by centrifugation (10 500 *g*, 10 min, 4 °C) and the supernatant was removed. Two further water washes were performed by the same method. After the third water wash the cell pellet was resuspended in 100 mM triethanolamine adjusted to pH 6.9 with maleic acid (TEA buffer). The cells were washed in the TEA buffer by stirring at 4 °C for 16 h. The wash volumes were one-twentieth of the culture volume in each case.

Preparation of cell lysate for purification of *C. thermocellum* chaperonins

It became clear that TEA extraction was not an efficient method for chaperonin extraction and that further chaperonins were liberated by sonication after the TEA extraction described above. To maximize the yield of chaperonins, the following protocol was used. After harvesting of the cells and one wash with water as described above, the cell pellet was sonicated (Heat Systems-Ultrasonics W-375 Sonicator) for 5 min (on ice, with a 50 % duty cycle) while resuspended in TEA buffer, and the cell debris removed by centrifugation (10 500 *g*, 30 min, 4 °C).

Protein assays

All of the protein assays described here were performed with the Bradford dye-binding assay [20] with BSA as a standard. Purified *C. thermocellum* Cpn60 was also quantified by the 205 nm absorbance method of Scopes [21] and showed good agreement (within 10 %) with the Bradford assay results.

SDS/PAGE

SDS/PAGE [22] was performed with a 10 % (w/v) resolving gel and a 4 % (w/v) stacking gel. Gradient gels (8–20 %, with 4 % stack) were used to reveal Cpn10.

N-terminal sequencing

Protein from the crude TEA wash was separated by SDS/PAGE, electroblotted on to poly(vinylidene difluoride) (PVDF; Millipore) membrane and the bands of interest excised. The sequence of the N-terminal amino acids was determined at the AFRC Microchemical Facility, Babraham, by automated Edman degradation with an Applied Biosystems 470A gas-phase sequencer [23].

Purified *C. thermocellum* Cpn60 (recombinant and authentic) and purified authentic *C. thermocellum* Cpn10 were sequenced directly by Roman Hlodan (University of Kent, Protein Science Facility) by automated Edman degradation with an Applied Biosystems 492 protein sequencer. Repetitive yields were more than 90 % in every case.

Western blotting

Protein was separated by SDS/PAGE and electroblotted on to PVDF membrane. Electroblotting was performed in a Hoeffer wet-tank system [24] at 150 mA for 2 h. The membrane was blocked with 3 % (w/v) dried milk (Marvel) in PBS supplemented with 1 % (v/v) polyoxyethylene sorbitan monolaurate (PBS/Tween). The membrane was washed (three times for 5 min each in PBS) and incubated in the primary antibody (diluted in PBS) at room temperature, with shaking for 1 h. The wash and incubation was repeated as above, with the secondary antibody solution. The blots were visualized with the Amersham ECL kit in accordance with the manufacturer's instructions.

The antibodies used were as follows.

Primary antibody

Either 1:1000 dilution rabbit anti-(*E. coli* GroEL) (a gift from John Ellis, University of Warwick) or 1:3000 dilution rabbit anti-(*E. coli* GroES) (a gift from Saskia van der Vies, University of Geneva).

Secondary antibody

Goat anti-(rabbit IgG), horseradish peroxidase conjugate, 1:1000 dilution.

Native molecular mass of *C. thermocellum* chaperonins

A Superose 6 gel-filtration column was equilibrated in 20 mM Tris buffer, pH 7.5, containing 0.1 M NaCl and calibrated with bovine thyroglobulin (669 kDa), equine apoferritin (420 kDa), yeast alcohol dehydrogenase (150 kDa) and BSA (67 kDa).

A crude sonicated TEA extract was concentrated by precipitation with 50 %-saturated ammonium sulphate (a procedure that selectively enriches for the chaperonins). The precipitate was redissolved in gel-filtration buffer and loaded on the Superose 6 column. Fractions (1 ml) were collected and the presence of chaperonins was determined by SDS/PAGE. The molecular mass of the chaperonins was estimated by comparison with the molecular mass standard proteins described above.

Purification of Cpn60

The crude cell-free extract (either a TEA buffer wash or sonicated cell lysate) was concentrated by ammonium sulphate precipitation (50 %-saturated ammonium sulphate) and dialysed against 20 mM Tris buffer, pH 7.5, containing 10 mM KCl and 10 mM MgCl₂. An ATP-agarose (Sigma) column (5 ml bed volume) was equilibrated in the buffer described above and the dialysed protein solution applied to the column. The column was washed successively with equilibration buffer (5 ml), 1 M NaCl in equilibration buffer (10 ml) and 3 mM ATP in equilibration buffer (15 ml). The eluate was collected in 5 ml fractions.

The ATP-agarose eluate was concentrated (Filtron 10 kDa, membrane filter) to approx. 1.0 ml in volume and loaded on to a Sephacryl S-200 gel-filtration column (120 ml matrix volume, 16 mm diameter). The column was developed with 20 mM Tris buffer, pH 7.5, containing 0.1 M NaCl at 2.0 ml/min. Fractions were collected and initially analysed by absorbance at 280 nm; those showing evidence of protein were analysed by SDS/PAGE. Pure Cpn60 was pooled and concentrated (Filtron 10 kDa membrane filter).

Purification of Cpn10

The unbound fraction from the ATP-agarose column was concentrated (Filtron 10 kDa membrane) and loaded on to a Sephacryl S-200 column (120 ml bed volume, 0.4 ml/min in 20 mM Tris buffer, pH 7.5, containing 0.1 M NaCl). Fractions containing Cpn10 (identified by SDS/PAGE) were pooled, concentrated as above and diafiltered into 20 mM Tris buffer, pH 7.5. The concentrate was loaded on to a Mono-Q FPLC column and eluted with a linear NaCl gradient (0–1 M in equilibration buffer). Fractions containing Cpn10 and the 9 kDa contaminant were pooled, diafiltered into equilibration buffer and chromatographed again with a gradient optimized to remove the contaminant. After the Mono-Q chromatography the fractions free of the contaminant were adjusted to 2 M ammonium sulphate (by addition of the solid) and loaded on to an Alkyl-Superose FPLC column. The proteins were eluted by a linear ammonium sulphate gradient (2–0 M). Pure Cpn10 was

eluted in the unbound fraction and in the early part of the gradient (2–1.5 M ammonium sulphate).

Purification of recombinant *C. thermocellum* Cpn60

The *C. thermocellum* Cpn60 was expressed in *E. coli* as a GST/Cpn60 fusion protein to facilitate separation from *E. coli* Cpn60. *E. coli* (JM83, pGGr2) [25] were grown to a D_{600} of between 0.4 and 0.5 and induced with 0.5 mM isopropyl thio-galactoside. After 3 h of further growth the cells were harvested, resuspended in PBS containing 1% Triton X-100 and sonicated. The soluble fraction was applied to a 10 ml glutathione-agarose column [equilibrated in PBS containing 1% (w/v) Triton X-100] at 0.5 ml/min. After washing with PBS (two column volumes) the bound fusion protein was eluted with 10 mM reduced glutathione in 50 mM Tris buffer, pH 8.0 (two column volumes). The fusion protein was cleaved by incubation with thrombin (2 NIH units per mg of fusion protein, 2 h, 25 °C). The cleavage was stopped by addition of PMSF to a final concentration of 100 µg/ml. The Cpn60 moiety was purified in the same way as the authentic *C. thermocellum* Cpn60 described above. Typically between 0.5 and 1 mg of pure recombinant Cpn60 was produced per litre of culture.

Electron microscopy

Carbon was evaporated on to grids with an Edwards coating unit. Protein solution (5 µl) was allowed to absorb on the grid for 15 s, the excess being removed by blotting the edge of the grid with filter paper. A drop of 1% (w/v) uranyl acetate was applied to each grid and the excess removed. The grids were allowed to dry and were observed with a Philips 410 transmission electron microscope at 80 keV.

Image analysis by rotational enhancement

The electron microscope negatives were used to focus a clear, enlarged image of the ring-shaped complex on to photographic paper attached to a turntable. To test for n -fold symmetry, exposures were made after turning the turntable repeatedly through $360/n^\circ$; in this case $n = 6, 7$ or 8 .

ATPase assays

The malachite green phosphate assay [26] was scaled down to allow smaller quantities of protein to be used; the absorbance readings were made with a microtitre plate reader at 620 nm. Sigma phosphate standard solution was diluted to give a linear standard curve from 0 to 200 µM.

For the temperature optimum measurements, 0.45 µg of Cpn60 were incubated in 20 µl buffer containing 20 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 10 mM KCl and 1 mM ATP for 20 min at the indicated temperatures and cooled on ice before phosphate determination.

For the temperature stability measurements, the *C. thermocellum* Cpn60 and *E. coli* GroEL were diluted in the buffer described above with ATP excluded. The samples were then incubated at 70 °C, with portions taken for assay at the indicated times. The portions were cooled on ice, ATP added to 1 mM and incubated at 37 °C (*E. coli* GroEL) or 60 °C (*C. thermocellum* Cpn60) for 20 min before phosphate determination.

The inhibitory effect of *E. coli* GroES on the ATPase activity of *C. thermocellum* Cpn60 was investigated by performing ATPase assays on mixtures of the purified proteins. Samples were prepared containing either *C. thermocellum* authentic or recombinant Cpn60 with different molar ratios of *E. coli* Cpn10

(Epicentre Technologies). The samples were then incubated with ATP for 1 h at 40 °C (this temperature was chosen to ensure stability of the *E. coli* Cpn10 throughout the incubation). The samples were assayed for phosphate as described above. Blank incubations were performed containing identical concentrations of Cpn60/10 but in the absence of ATP. Controls were also incubated containing ATP in the absence of Cpn60/10. The ATPase activity was plotted against the molar ratio of (Cpn10)₇ to (Cpn60)₁₄. All k_{cat} values were calculated per active site, i.e. per Cpn60 monomer.

RESULTS

Protein extraction

The *C. thermocellum* Cpn60 and Cpn10 proteins were first detected and identified (by N-terminal sequencing, see below) in a buffer wash (TEA) of the cell pellet from a stationary-phase culture (Figure 1). The distinctive SDS/PAGE profile of this buffer wash showed that protein bands at 65 and 12 kDa were the predominant proteins extracted. Optimum extraction of these proteins required sonication of the cell pellet, but identical purification protocols were effective regardless of the extraction method.

The 65 and 12 kDa proteins are homologues of GroEL and GroES

After concentration of the TEA extract, it was resolved by SDS/PAGE and the predominant proteins were blotted on to PVDF membrane and sequenced. The 65 and 12 kDa proteins were identified by comparison of the N-terminal amino acids with known sequences (Figure 2). The sequences of the 65 and 12 kDa proteins showed significant similarity to proteins in the GroEL and GroES chaperonin family.

The identity of the 65 and 12 kDa proteins was further confirmed by their cross-reaction (in both crude and pure forms) with antibodies raised against *E. coli* GroEL and GroES respectively (Figure 3).

Purification of Cpn60

The TEA buffer extract or the sonicated *C. thermocellum* cell extract was concentrated and applied to an ATP-agarose

Figure 1 ATP-affinity purification of *C. thermocellum* Cpn60: SDS/PAGE analysis of the purification fractions on an 8–20% (w/v) gradient gel revealed by staining with Coomassie Blue

A concentrated TEA buffer extract (lane 1) was applied to an ATP-agarose affinity column in 20 mM Tris buffer, pH 7.5, containing 10 mM KCl and 10 mM MgCl₂. The unbound fraction (lane 2), equilibration buffer wash (lane 3) and salt wash (lane 4) contained negligible Cpn60. Cpn60 was eluted in 2 column volumes of 5 mM ATP (lanes 5 and 6). The lane labelled M contained molecular-mass marker proteins.

Figure 2 N-terminal sequence of *C. thermocellum* chaperonins

The N-terminal amino-acid sequence of *C. thermocellum* Cpn60 (upper panel) and Cpn10 (lower panel) are shown aligned with homologous sequences from other organisms. The boxed residues are identical with the *C. thermocellum* cDNA sequence [25]. Capital letters indicate residues with a high degree of certainty, small letters indicate residues named with less certainty, X indicates that no identification was possible for this residue. Notes: A1, native protein from *C. thermocellum* crude TEA extract, resolved by SDS/PAGE, transferred to PVDF and sequenced at the Microchemical Facility of the BBSRC Babraham Institute (the initial identification of the *C. thermocellum* chaperonins was made from these sequences); A2, native protein from *C. thermocellum* purified to homogeneity as described in the Materials and methods section and sequenced directly by Roman Hlodan, University of Kent Protein Science Facility; R, recombinant *C. thermocellum* Cpn60 from *E. coli* (JM83, pGGR2), purified as described in the Materials and methods section and sequenced directly by Roman Hlodan, University of Kent Protein Science Facility (underlined residues were remnants of the thrombin cleavage site engineered into the fusion protein [25]); C, translation of the *C. thermocellum* cloned DNA sequence [25], EMBL accession number Z68137 (this includes both Cpn60 and Cpn 10 sequences).

column (in the presence of K⁺ and Mg²⁺). The Cpn60 bound tightly to the column; loosely bound proteins were removed from the ATP-agarose column by washing with 1 M NaCl and the Cpn60 was then eluted with 5 mM ATP.

The ATP washes showed a single band of 65 kDa on SDS/PAGE (Figure 1). This material proved to be sufficiently pure for electron microscopy, but the Cpn60 was purified further by gel filtration on Sephacryl S-200 before use in ATPase assays. The Cpn60 was eluted in the void volume from the S-200 column, indicating that the chaperonin was present in an oligomeric form under native conditions. In a typical purification, *C. thermocellum* cell-free extract containing 39 mg of protein yielded 0.4 mg of pure Cpn60.

Figure 3 Western blotting of *C. thermocellum* chaperonins

Lanes 1, 3, 5 and 6, crude TEA buffer extract (see Figure 1); lanes 2 and 4, purified *C. thermocellum* Cpn60; lanes 7 and 8, purified *C. thermocellum* Cpn10. Lanes 1, 2, 5 and 7 were stained with Coomassie Blue; lanes 3 and 4 were Western blotted with antibodies against *E. coli* GroEL; lanes 6 and 8 were Western blotted with antibodies against *E. coli* GroES. Antibodies against *E. coli* GroEL and GroES were kindly provided by John Ellis (University of Warwick) and Saskia van der Vies (University of Geneva) respectively.

Figure 4 Purification of *C. thermocellum* Cpn10

Cpn10-containing fractions after each stage of purification: (a) ATP-agarose, unbound; (b) after Sephacryl S-200 gel filtration; (c) after FPLC on Mono-Q; (d) after FPLC on Alkyl-Superose. Gels in (a), (b) and (c) were stained with Coomassie Blue. The fractions shown in (d) contained high concentrations of ammonium sulphate and so were diluted 1:6 before electrophoresis and silver staining.

Purification of Cpn10

The unbound fraction from the ATP-agarose column was free of Cpn60 and so was a convenient starting material for the purification of Cpn10. Gel filtration on Sephacryl S-200 (Figure 4b) removed some high-molecular-mass proteins, but several other protein contaminants were still present.

Mono-Q FPLC (Figure 4c) separated the Cpn10 from most contaminants, but its elution range (0.27–0.45 M NaCl) overlapped with that of a major contaminant (molecular mass 9 kDa) that was eluted between 0.2 and 0.35 M NaCl. A second Mono-Q run was necessary for complete removal of this contaminant (results not shown).

A trace contaminant (45 kDa) was still present and bound strongly to Alkyl-Superose. The Cpn10 bound only weakly and was present in the unbound fraction and early part of the elution (2–1.5 M ammonium sulphate). After concentration, the Cpn10-containing fractions seemed to be pure on SDS/PAGE (Figure 4d and Figure 3, lane 6). A typical *C. thermocellum* cell-free extract containing 40 mg of protein yielded 30 µg of pure Cpn10.

Native molecular mass of the *C. thermocellum* chaperonins

A sonicated cell extract was concentrated and selectively enriched for chaperonins by ammonium sulphate precipitation (50% saturation). This material was then fractionated on a Superose 6

Figure 5 Native molecular mass of *C. thermocellum* chaperonins

The chaperonins from a crude TEA extract were selectively enriched by ammonium sulphate precipitation (50% saturation) and fractionated on a Superose 6 gel-filtration column. The elution volume of the chaperonins was compared with a range of molecular mass standard proteins. The elution position of the standard proteins is shown beneath the gel: A, BSA (67 kDa); D, yeast alcohol dehydrogenase (150 kDa); F, apo-ferritin (450 kDa); T, bovine thyroglobulin (690 kDa). The Cpn60 peak centres on 420 kDa, suggesting that it migrates as a heptamer under these conditions. The lane labelled M contained SDS/PAGE molecular-mass standards (94, 67, 43, 30, 20 and 14 kDa).

gel-filtration column at room temperature and the mobility of the chaperonins compared with a range of molecular mass standards. SDS/PAGE of the fractions showed that Cpn60 was eluted in a broad peak with about the same elution volume as the apo-ferritin standard (Figure 5). This suggests that the Cpn60 complex under these conditions (without added adenine nucleotides) has a molecular mass of approx. 450 kDa, which is the expected molecular mass of a heptameric Cpn60 complex. A tetradecameric Cpn60 complex has a molecular mass of approx. 850 kDa, which would be eluted earlier than the thyroglobulin

Figure 6 Electron microscopy of *C. thermocellum* Cpn60

The electron micrograph shows that *C. thermocellum* forms toroidal complexes of similar structure to *E. coli* GroEL (one example is indicated by the thin arrow). Most of the complexes are orientated with the plane of the toroid parallel to the grid; in this orientation it is impossible to distinguish between heptamers and tetradecamers. A few complexes are seen in side view (indicated by a wide arrow), and these seem to consist of two stacked toroids as seen in *E. coli* GroEL.

Figure 7 *C. thermocellum* Cpn60 shows 7-fold rotational symmetry

An electron micrograph showing the top view of a *C. thermocellum* Cpn60 complex was analysed for rotational symmetry. The image was projected on to photographic paper and several (n) exposures were made with a rotation of $360/n^\circ$ between successive exposures. When n is equivalent to the symmetry of the complex, the multiple exposures overlay exactly and reinforce the image of the subunits, giving a clear well-defined picture. When n is not equivalent to the symmetry of the complex the subunits do not overlay exactly, giving a poorly defined final image. In this case the clearest image was seen under 7-fold rotation, which suggests that the complex has 7-fold symmetry.

standard (650 kDa). In this analysis no protein was detectable with an elution volume less than that of thyroglobulin.

The midpoint of the Cpn10 elution peak coincided with the approximate elution position of the BSA standard (67 kDa). This suggests that the *C. thermocellum* Cpn10, like *E. coli* GroES, is present as an oligomeric (probably heptameric) complex under native conditions.

Electron microscopy

Electron microscopy of the purified Cpn60 showed ring-shaped complexes (Figure 6), which (when analysed by rotational symmetry enhancement) showed 7-fold symmetry (Figure 7). The diameter of the complexes was 10.5 nm (mean of 20 measured).

This ring-shaped appearance is a characteristic of the Cpn60 protein complex when seen in top view. The majority of the observed complexes (at least 95%) were seen in top view and may represent either heptamers or tetradecamers, as these cannot be distinguished when seen in this orientation. There were a few 'brick'-shaped objects present (Figure 6), which were approx. 15 nm × 12 nm. These complexes showed the four transverse stripes that are thought to indicate the domain structure of the Cpn60 monomers within a tetradecamer [27–29].

ATPase activity of Cpn60

The ATPase activities of the authentic and recombinant *C. thermocellum* Cpn60 proteins were determined across a range of temperatures between 30 and 80 °C (Figure 8). Both preparations

Figure 8 Effect of temperature on ATPase activity of authentic and recombinant *C. thermocellum* Cpn60

Authentic and recombinant *C. thermocellum* Cpn60 were incubated at the indicated temperature for 20 min in buffer containing 20 mM Hepes, pH 7.3, 10 mM KCl, 10 mM $MgCl_2$ and 1 mM ATP. The phosphate concentration was determined at the end of the incubation by using the Malachite Green phosphate assay. At the optimum growth temperature for *C. thermocellum* the Cpn60 shows ATPase activity similar to that of *E. coli* GroEL at 37 °C. The assays were performed in duplicate; bars indicate the range of the duplicates.

showed low activity at 30 °C, which increased with temperature. Authentic and recombinant *C. thermocellum* Cpn60 showed maximum activity (of approximately $0.07\ s^{-1}$) between 60 and 80 °C. Within the optimum temperature range for *C. thermocellum* growth (55–65 °C), the ATPase activity of both authentic and recombinant *C. thermocellum* Cpn60 was comparable with that of *E. coli* GroEL at 37 °C.

The ATPase activity was used as an indicator of the thermostability of the *C. thermocellum* and *E. coli* Cpn60. The *C. thermocellum* (authentic and recombinant) Cpn60 and *E. coli* Cpn60 were incubated at 70 °C for various times. The residual ATPase activity was then determined by assays performed at the optimum growth temperature of the source organism (60 °C for *C. thermocellum* Cpn60; 35 °C for *E. coli* Cpn60). Both the recombinant and authentic *C. thermocellum* Cpn60 were more stable at 70 °C than the *E. coli* Cpn60 (Figure 9). The half-life of the *E. coli* Cpn60 ATPase activity at 70 °C was approx. 30 min; less than 5% activity was retained after 90 min. The ATPase activity of the chaperonins derived from *C. thermocellum* showed a half-life of more than 90 min at 70 °C. The loss of activity with duration of heat treatment seemed to follow an exponential decay.

Inhibition of *C. thermocellum* Cpn60 ATPase by *E. coli* Cpn10

Experiments to investigate the inhibition of authentic *C. thermocellum* Cpn60 by *C. thermocellum* Cpn10 showed that, although the Cpn10 was apparently pure by SDS/PAGE, it retained a contaminating ATPase activity that made interpretation of these experiments impossible.

Inhibition by *E. coli* GroES was then studied with a range of molar ratios of authentic *C. thermocellum* Cpn60 and commercially available purified *E. coli* GroES. A temperature of 40 °C was chosen to give detectable ATPase activity from the thermophilic Cpn60 and to prevent denaturation of the mesophilic GroES. The ATPase activity of the Cpn60 decreased with increasing Cpn10 concentration until a ratio of one (Cpn10)₇

Figure 9 Thermostability of Cpn60 from different sources

The ATPase activity was used as an indicator of the thermostability of Cpn60 from different sources. Recombinant and authentic *C. thermocellum* Cpn60 and *E. coli* Cpn60 were incubated at 70 °C for the durations shown. The ATPase activity was then assayed by a 20 min incubation at 60 °C (*C. thermocellum* Cpn60) or 35 °C (*E. coli* Cpn60) with detection of phosphate by the Malachite Green assay. The ATPase activity was expressed as a percentage of the original activity before heat treatment and is shown plotted on a semilogarithmic scale (because the activity appeared to follow an exponential decay). ATPase assays were performed on 20 μ l samples in a buffer containing 20 mM Hepes, pH 7.3, 10 mM KCl, 10 mM $MgCl_2$ and 1 mM ATP. The assays were performed in duplicate; bars indicate the range of the duplicates.

Figure 10 Inhibition of *C. thermocellum* Cpn60 by *E. coli* Cpn10

The ATPase activities of both authentic and recombinant Cpn60 were inhibited by *E. coli* GroES. Maximum inhibition occurred when the Cpn60-to-GroES ratio (based on monomers) was 2:1. The assays were performed in duplicate; bars indicate the range of the duplicates.

complex per (Cpn60)₁₄ complex was reached (a molar ratio of 1:2 Cpn10 to Cpn60 monomers). A further doubling of the Cpn10 concentration caused no significant change in ATPase activity. The final, partially inhibited ATPase activity was approx. 40 % that of the uninhibited enzyme (Figure 10).

A similar experiment performed with the recombinant *C. thermocellum* Cpn60 showed the same stoichiometry of inhibition by *E. coli* Cpn10, although the ATPase activity under conditions of maximum inhibition was approx. 60 % of the original activity (Figure 10).

DISCUSSION

Evidence presented here indicates that we have isolated Cpn60 and Cpn10 homologues from *Clostridium thermocellum* cells. The N-terminal amino-acid sequences show very high degrees of identity with other Cpn60/10 proteins. The similarity (Figure 2) is closest to Cpn60/10 proteins from other Gram-positive bacteria (more than 90 % identity with thermophiles *PS3* and *Thermoanaerobacter brockii* [7,30]) but, as would be expected in such a highly conserved protein family, there is also similarity to Gram-negative bacterial proteins (more than 60 % identity with *E. coli* [1]) and mitochondrial (more than 50 % identity with rat hsp60 [31]) proteins.

The N-termini of the Cpn60 and Cpn10 proteins were sequenced both from the crude extract and from the purified samples and show good agreement with the cDNA sequence (EMBL accession number Z68137) determined in a complementary study [25]. The identity of these proteins was further confirmed by cross-reaction with antibodies raised against the homologous proteins from *E. coli* (Figure 3).

The purification procedure for Cpn60 used the ATP-binding properties of the protein and was an early indication that the Cpn60 was a functional ATPase (Figure 1). The purification of Cpn10 was less simple, but was achieved by gel-filtration, ion-exchange and hydrophobic interaction chromatographies.

The electron micrographs of the purified Cpn60 (Figure 6) showed images very similar to the GroEL complexes isolated from *E. coli* [32,33], *Bacillus subtilis* [34] and *Paracoccus denitrificans* [27]. Most of the Cpn60 complexes seen were orientated perpendicular to the grid so that the 7-fold symmetrical ring was seen; this makes differentiation between heptamers and tetradecamers impossible. Small numbers of complexes were seen showing the characteristic side view of the Cpn60 tetradecamer, but because the great majority of complexes were seen in top view, no meaningful analysis of the relative frequency of tetradecamers or heptamers is possible. The dimensions of the *C. thermocellum* complex in side view (15 nm × 12 nm) are similar to those of other bacterial Cpn60 complexes [27,29,34]. The *C. thermocellum* Cpn60 complex did not show any evidence of the bullet shape seen in *Thermus thermophilus* [27,35], which was interpreted as showing the holo-chaperonin consisting of Cpn60 and Cpn10. The absence of Cpn10 in our purified sample was confirmed by SDS/PAGE.

Gel filtration (Figure 5) showed that, under the conditions used, heptameric complexes were the predominant Cpn60 species present. Species with the mass of tetradecamers were not detected by this method, but this does not eliminate the possibility that tetradecamers, are significant under physiological conditions. The fact that some tetradecamers are observed by electron microscopy indicates that the *C. thermocellum* Cpn60 can form tetradecamers, and the stoichiometry of interaction with *E. coli* GroES (see below) suggests that tetradecamers are the catalytically active species.

In *E. coli* GroEL, where the high-resolution structure has been

determined [29], the inter-subunit binding generating the toroid of seven subunits is considerably more extensive and stronger than the predominantly hydrophobic inter-toroid interactions that give rise to the tetradecameric complex. Work on a number of other Cpn60 systems suggests that heptamer and tetradecamer species may be in facile equilibrium, but that the tetradecamer is always the functional form. A thermostable chaperonin from *Thermoanaerobacter brockii* [30] has recently been characterized that is apparently heptameric at room temperature and at 65 °C. Under conditions where productive refolding of proteins is expected (i.e. in the presence of Cpn10 and MgATP²⁻) the heptamers dimerize and associate with Cpn10 to form an active holo-chaperonin complex [36]. In *T. thermophilus* disaggregation of the active tetradecameric Cpn60 into heptamers has also been documented [37]. These results in other systems suggest that the heptameric particles that we have detected from *C. thermocellum* may be due to disaggregation of tetradecamers caused either by a low temperature effect or by some other combination of conditions occurring during the purification and/or analysis. The detection of tetradecameric particles by electron microscopy suggests that there is an equilibrium between tetradecamers and heptamers, although we have not yet defined the conditions effecting this equilibrium.

The ATPase activity of the *C. thermocellum* Cpn60 shows the chaperonin to be considerably more stable at high temperature than the *E. coli* homologue. This property may be useful in both investigating and optimizing the folding of thermostable proteins. The optimum ATPase activity is very similar to that seen in mesophilic chaperonins but the temperature optimum is shifted to correspond to the higher growth temperature of the organism.

The inhibition of the *C. thermocellum* Cpn60 ATPase by *E. coli* GroES shows similar characteristics (in terms of stoichiometry and extent of inhibition) to that previously shown in the homologous *E. coli* GroEL/ES system. The stoichiometry of this interaction, indicating maximum inhibition at a molar ratio (Cpn10 to Cpn60) of 1:2, is further evidence that the active form of the *C. thermocellum* Cpn60 might be a tetradecamer rather than the heptamer seen during gel filtration at room temperature. The functional interaction between heterologous Cpn60 and GroES demonstrates that the widely documented sequence homology of the chaperonin family is matched by widespread functional homology.

C. thermocellum exports large amounts of protein in the form of cellulases, forming the highly organized cellulosome complex (reviewed in [15,38]). In a fermentation containing partly degraded cellulose, a complicated system exists where bacterial cells are bound to cellulose fibres through the cellulose-binding affinity of the cellulosome. A TEA buffer wash has previously been used to prepare the cellulosome of *C. thermocellum* for electron microscopy. The bacterial cells were removed from the cellulose by washing in TEA buffer, leaving the cellulosome attached to the cellulose for analysis by electron microscopy [12,39].

In our study, the TEA buffer wash of cells grown on cellobiose (a soluble substrate) contained two predominant proteins, identified by N-terminal sequencing as Cpn60 and Cpn10 homologues. Subsequently it was found that sonication of the cell pellet after extensive washing with TEA buffer caused further release of these chaperonins. These proteins could be purified by identical protocols regardless of whether they were released by extraction with TEA buffer or by sonication.

It would be interesting to speculate on a possible role for the *C. thermocellum* chaperonin system in the production and translocation of the cellulosomal components. At present the mode of transport of the cellulosomal proteins through the cell membrane and cell wall is only poorly understood. The

cellulosome is known to be extremely robust; purification of individual components requires denaturation of the proteins [40], and recent work [16,17] suggests that the complex might be highly organized with a receptor/ligand association between CipA, a non-catalytic scaffold protein, and the catalytic components. The site of assembly of the cellulosome has not been confirmed, but localization on the cell surface seems to be mediated by its co-assembly with proteins showing homology with bacterial S-layer components [18]. Owing to the size and nature of the cellulosome, transport of the entire complex through the membrane is unlikely. Therefore assembly must occur either within the peptidoglycan matrix or at the cell surface. As the cellulosome components show the propensity to associate with CipA spontaneously, some mechanism must be present to prevent premature assembly of the complex within the cell. In other organisms the chaperonin system has been shown to fulfil this role with several other protein complexes and multimeric enzymes. Further work is required to investigate this aspect of chaperonin function in *C. thermocellum*.

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