The inter-ring arrangement of the cytosolic chaperonin CCT

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The eukaryotic cytosolic chaperonin CCT (chaperonin containing TCP-1) is the most complex of all chaperonins—an oligomeric structure built from two identical rings, each composed of single copies of eight different subunits. The arrangement of the eight subunits within each ring has been characterised for some time, but the phasing between the two rings remains unknown. Here, three-dimensional reconstructions generated by cryoelectron microscopy of complexes between CCT and either of two different monoclonal antibodies that react specifically with the CCTε and CCTδ subunits have been used to determine the phasing between the two chaperonin rings. The inter-ring arrangement is such that up/down inter-ring communication always involves two different CCT subunits in all eight positions, and the group of subunits concerned with the initiation and completion of the folding cycle cluster together both in the intra- and inter-ring arrangement. This supports a sequential mechanism of conformational changes between the two interacting rings.

Keywords: molecular chaperones; chaperonin; CCT; protein folding; electron microscopy

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INTRODUCTION

Chaperonins are oligomeric proteins, usually composed of two rings placed back-to-back, each enclosing a cavity in which protein folding occurs. They are divided into two groups: those

found in eubacteria or endosymbiotic organelles (Bukau *et al*, 2000), and those in archaea and the eukaryotic cytosol (Gutsche *et al*, 1999; Valpuesta *et al*, 2005). The studies carried out with the group I paradigm GroEL from *Escherichia coli* have shown that this chaperonin is able to recognise and act on a large variety of unfolded proteins, relying mostly on hydrophobic interactions between the apical domains of the chaperonin and the unfolded polypeptide. The closure of the chamber provides the unfolded polypeptide with an isolated environment in which it can reach the native conformation without any unwanted interaction. Conversely, the group II chaperonin CCT (chaperonin containing TCP-1) recognizes a more defined set of substrates that already have a certain degree of conformational maturity and makes use of coordinated conformational changes that generate the closure of its cavity to force the folding of the protein, as shown for the actins and tubulins (Llorca *et al*, 2001). The different behaviour might rely on the differences in intra- and inter-ring allosteric signalling between the two chaperonins (Horovitz & Willison, 2005) and in their subunit composition. GroEL is composed of two staggered, homo-heptameric rings, whereas each CCT ring is composed of eight different subunits (CCTα, CCTβ, CCTγ, CCTδ, CCTε, CCTζ, CCTη and CCTθ, corresponding to CCT1–CCT8 in yeast) that have specific interactions with the unfolded protein, and their equatorial domains interact with only one other subunit in the opposite ring. The arrangement of the CCT subunits in a ring is known and follows a unique pattern (Liou & Willison, 1997), but the arrangement between the two rings has not been determined, although several modes of ring-interaction have been proposed by others (Miller *et al*, 2006) on the basis of the data available from our reconstruction of CCT–antibody complexes (Grantham *et al*, 2000). In this work, the phasing of the two CCT rings has been solved by carrying out two different three-dimensional reconstructions of CCT complexed to two different monoclonal antibodies that bind specifically to CCTε and CCTδ subunits.

RESULTS AND DISCUSSION

Binding experiments with the CCTε MAb εAD1

As discussed above, the intra-ring arrangement and handedness of CCT is known (Liou & Willison, 1997; Fig 1A) following

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Fig 1 | Binding of the CCTε monoclonal antibody εAD1 to CCT. (A) The intra-ring subunit arrangement, as determined biochemically (Liou & Willison, 1997). The arrangement describes the ring observed from the end of the particle, as shown by immunomicroscopy experiments (Llorca *et al*, 1999, 2000). The asterisks and arrows indicate the genetic evidence for the hierarchical changes in the CCT ring using ATP-binding mutants of the yeast homologues of CCTα, CCTδ, CCTβ and CCTζ (Lin & Sherman, 1997). (B) Native gel shift assay of CCT in the absence or presence of εAD1 monoclonal antibody. The positions of εAD1, CCT and double antibody shifted CCT complexes (CCTs) are indicated. (C–E) Two-dimensional average images from negatively-stained particles. (C) Two-dimensional side view average image (in contour lines) of the CCT–εAD1 complex. Arrows point to the antibody mass. (D) Two-dimensional average image of the end-on view of the same complex. (E) Two-dimensional average image of the end-on view of the complex formed between CCT, the intact CCTδ monoclonal antibody 8g and the Fab fragment of the CCTε εAD1 antibody. The CCT subunits involved with antibody binding (CCTδ and CCTε) are indicated in greek letters. εAD1, epsilon apical domain 1; CCT, chaperonin containing TCP-1.

immunomicroscopy experiments carried out with complexes between CCT and actin and tubulin, or domains of these proteins (Llorca *et al*, 1999, 2000). From the known intra-ring arrangement of subunits, we decided to generate three-dimensional reconstructions of CCT–MAb complexes with MAbs that reacted against specific subunits. This approach detected the relative position of individual subunits in the double-ring structure, which determined the phase between the CCT rings. A monoclonal antibody recognising an epitope located at the tip of the helical extension of mouse CCTε apical domain (εAD1) was produced (see supplementary Fig S1 online), and its ability to bind to bovine CCT was tested by using biochemical and electron microscopy methods. First, a native gel of the complex formed between CCT and εAD1 showed a shifted band that correlated with a double antibody-labelled chaperonin complex (Fig 1B). The electron microscopy of the CCT–εAD1 complexes showed the two typical views observed with apo-CCT—the circular end-on view and the orthogonal side view—showing the two-ring structure of the chaperonins. For the side view, 691 particles of CCT–εAD1 complexes were selected and processed. The average image showed the presence of a stain-excluding mass protruding from the apical domain of each of the two CCT rings (Fig 1C). The image confirms that the epitope of this antibody localizes in the top region of the apical domain and strengthens the model for the presence of only one CCTε subunit in both chaperonin rings,

as found with CCT–CCTα monoclonal antibody complexes (Grantham *et al*, 2000). Furthermore, 832 end-on particles, in which antibody molecules extended outside the CCT cavity and therefore could be visualised, were selected and processed. The average image of the CCT–εAD1 complex, in which only one of the chaperonin rings was stained and therefore contrasted, showed a stain-excluding mass protruding again from only one of the eight CCT subunits (the stain contrasts one of the chaperonin rings only; Fig 1D). An experiment was devised to confirm the model for the unique and relative positions of two of the CCT subunits in a ring. A Fab (fragment of antigen binding) fragment was generated from the εAD1 antibody and was incubated with CCT together with an intact IgG monoclonal antibody, MAb 8g, which reacts against the bottom part of the apical domain of the CCTδ subunit (Llorca *et al*, 1999). A total of 622 electron microscopy particles of the negatively stained complexes were selected and processed. The average image of the complex between CCT, the IgG CCTδ 8g antibody and the Fab fragment of the CCTε antibody (Fig 1E) showed two stain-excluding masses protruding from the CCT ring. The two masses—a larger one belonging to the full monoclonal antibody and a smaller one that corresponds to the Fab fragment—were separated by two intervening CCT subunits. This arrangement is consistent with the subunit topology obtained biochemically by Liou & Willison (1997), and with other immunomicroscopy

Fig 2 | Three-dimensional reconstruction generated by cryoelectron microscopy of the complex between CCT and the CCTε εAD1 monoclonal antibody. (A,B) Side and end-on views, respectively, of the CCT-εAD1 complex. In (A), the equatorial (E), intermediate (I) and apical (A) domains, and the part of the antibody reconstructed (Ab) are indicated and coloured. (C) Epitope localisation of εAD1, which interacts with residues Pro260-Tyr274 of CCTε. The binding domain is shown in red in the homologous region of the structure of the α-thermosome from *Thermoplasma acidophilum* (Ditzel *et al*, 1998; PDB 1A6D). As in (A) the regions marked E, I and A correspond, respectively, to the equatorial, intermediate and apical domains. εAD1, epsilon apical domain 1; CCT, chaperonin containing TCP-1.

experiments carried out with CCT-actin or CCT-tubulin complexes decorated with monoclonal antibodies (for a summary of these results, see Valpuesta *et al*, 2005).

3D reconstructions of two CCT-MAb complexes

Having confirmed utility of the εAD1 CCTε monoclonal antibody for electron microscopy purposes, complexes between CCT and this antibody were purified and frozen-hydrated for their observation by cryoelectron microscopy. A total of 10,294 particles were selected and processed as described in the Methods. The three-dimensional reconstruction obtained (Fig 2A,B) shows a structure composed of two CCT rings and two small masses that correspond to part of an antibody protruding from the apical domain of the CCTε subunit in each of the chaperonin rings and extending into the cavity. The volume also shows a clear asymmetric structure, with one of the CCT rings in a more open conformation than the other, despite the fact that no nucleotide is present in the solution (Fig 2A,B). The location of the antibody fits very well, at the resolution of this reconstruction, with the location of the epitope recognised by the εAD1 antibody in the tip of the helical extension located in the apical domain of CCTε (Fig 2C; supplementary Fig S3 online). Only a small portion of the antibody, the region closest to the chaperonin, has been reconstructed owing to the fact that the rest of the antibody is very flexible and much of the density appears to be averaged out during the reconstruction procedure. The three-dimensional reconstruction of the CCT-εAD1 complex shows that the two CCTε subunits are located near each other in the inter-ring arrangement, displaced by only one CCT subunit, clockwise as seen from the end of the particle (Figs 2A,B,4A; supplementary Fig S4 online).

To confirm the subunit arrangement shown in the volume of the CCT-εAD1 complex, a second three-dimensional reconstruction was carried out, this time between CCT and the CCTδ monoclonal antibody 8g (supplementary Fig S1 online; Llorca *et al*, 1999). A total of 5,422 particles were selected and processed as before to render the three-dimensional reconstruction depicted

in Fig 3, which shows a cylindrical structure with two small masses protruding from two CCT subunits from different rings, most probably belonging to the reconstructed part of the antibodies (Fig 3A,B). Again, the structure of the CCT-8g complex is asymmetric, with one of the chaperonin rings in a more open conformation than the other (Fig 3A,B). This asymmetry might be intrinsic or generated by the contact of one of the rings with the carbon film. The small part of the antibody that is reconstructed stems from the central part of the CCTδ subunit, which fits well with the position of the epitope that the 8g antibody recognizes (supplementary Fig S3 online; Llorca *et al*, 1999), located at the bottom of the apical domain, close to the intermediate domain (Fig 3C). As in the case of the CCT-εAD1 complexes, an asymmetry is observed between the apical domains of the two rings, with one ring in a more open conformation than the other (Figs 2,3), which might result in an apparent difference in the exact position of the of the antibody-binding site. In any case, the two CCTδ subunits are placed close to each other, displaced by one CCT subunit, anticlockwise as seen from the end of the reconstructed volume (Figs 3A,B,4B). The vorticity of the chaperonin structure accounts for the much greater difference in the separation of the antibody masses in CCT-8g compared with CCT-εAD1 despite the fact that CCTε and CCTδ are both offset by only one subunit in the inter-ring arrangement (compare Fig 4A, with B).

Implications of the CCT inter-subunit arrangement

Conformational changes in chaperonins are governed by allosteric signals that originate upon ATP binding/hydrolysis and occur at the intra- and inter-ring level (Horovitz & Willison, 2005). All the chaperonins seem to have negative inter-ring cooperativity, but their comparative behaviour with respect to intra-ring cooperativity is more complex (Horovitz & Willison, 2005). GroEL shows a clear positive intra-ring cooperativity so that the changes in all seven identical subunits are concerted, whereas CCT has a weak positive cooperativity and the allosteric transitions are sequential

Fig 3 | Three-dimensional reconstruction generated by cryoelectron microscopy of the complex between CCT and the CCTδ 8g monoclonal antibody. (A,B) Side and end-on views of the CCT-8g complex. The colouring and lettering are as in Fig 2A. (C) Epitope localisation of 8g, which interacts with residues Pro344-Ser358 of CCTδ (Llorca *et al*, 1999). As in Fig 2C, the binding domain is depicted in red in the homologous region of the structure of the α -thermosome from *Thermoplasma acidophilum*. CCT, chaperonin containing TCP-1.

Fig 4 | Inter-ring arrangement of the CCT oligomer. (A) Inter-ring arrangement of the CCTε subunits. Although separated by only one subunit (clockwise, as seen from the top), there is no apparent shift. (B) Inter-ring arrangement of the CCTδ subunits. Although separated by only one subunit (anticlockwise, as seen from the top), the apparent shift is of two subunits. (C) In the inter-ring arrangement, a model for the sequential intra- and inter-ring mechanism of CCT folding is depicted. The coloured subunits are those labelled in A and B. The linear order of the subunits is as determined by Liou & Willison (1997). The black circular arrows represent the intra-ring sequential changes determined genetically by Lin & Sherman (1997). The asterisks indicate the putative inter-ring signalling between the first and last intra-ring subunits, lying opposite to each other in the inter-ring interface. The vertical arrows point to a simple inter-ring signalling mechanism in which CCTε and CCTζ could be, respectively, the first and last subunit to undergo the intra-ring sequential changes. CCT, chaperonin containing TCP-1.

(Kafri & Horovitz, 2003). This has been confirmed in part by electron microscopy analysis of CCT at various ATP concentrations, showing that changes occur sequentially, from the CCTα/η/δ/θ region to the CCTβ subunit (Rivenzon-Segal *et al*, 2005). Previous classical genetic studies carried out with ATP-binding site mutants in several yeast CCT subunits (Lin & Sherman, 1997), showed that ATP binding/hydrolysis occurs in a hierarchical manner (Fig 1A), starting in CCTα and ending in CCTζ. Combining these data with the intra-ring subunit arrangement obtained by using a biochemical approach (Liou & Willison, 1997) suggests a sequential cascade of ATP-dependent events starting in CCTα and ending in CCTβ/ζ. This leaves CCTε in an undetermined position in the sequential cycle as, according to the available data, it could be the first or the last CCT subunit to undergo such conformational change (Fig 1A). The two three-dimensional reconstructions presented here (Figs 2,3) show a unique inter-ring combination (Fig 4C). It is striking that CCTα, the top member of the ATP hierarchy (Lin & Sherman, 1997), and CCTβ, the last member of the ATP concentration-dependent sequential conformational hierarchy (Rivenzon-Segal *et al*, 2005), lie opposed at the ring interface. This suggests a solution in which the group of subunits concerned with the initiation and completion of the folding cycle cluster together in the intra- and inter-ring arrangement (see subunits marked by asterisks in Fig 4C). A simpler model could involve intra-ring initiation in CCTε and completion in CCTζ, both of which are directly opposed in the inter-ring connection (see arrows in Fig 4C). Once an intra-ring cycle has been completed, CCTζ might signal to CCTε in the opposite ring for a new cycle to be initiated. In any of the various possibilities, inter-ring signalling between the opposite subunits would ensure that a ring only acts after the other one has undergone its sequential changes and that the two CCT rings of this nanomachine always work in alternate modes.

METHODS

Biochemical methods. CCT was purified from bovine testis according to Martín-Benito *et al* (2002). The CCTδ 8g monoclonal antibody was prepared as described by Llorca *et al* (1999) and the

CCTε εAD1 antibody as described by Llorca *et al* (2000). The epitope recognised by εAD1 (residues Pro260–Tyr274) was determined by screening a set of solid-phase 15-mer peptides (Hynes & Willison, 2000) scanning the primary sequence of the mouse CCTε apical domain.

Binding of εAD1 to CCT was checked by a gel shift assay using the methods described by Liou *et al* (1998) for measuring single- and double-antibody-bound CCT complexes. A 0.3 µg portion of CCT was incubated in the absence or presence of 1.14 µg of the εAD1 antibody. Samples were made up to 10 µl with PBS and incubated on ice for 40 min. A 1.5 µl volume of native loading buffer was added and the whole sample run on a 6% native gel stained with Coomassie blue.

The Fab fragment of εAD1 was prepared by incubating 0.45 mg of εAD1 with 4.5 µg papain in the presence of 100 mM sodium acetate (pH 5.5), 50 mM cysteine and 1 mM EDTA overnight at 37°C. A one-tenth volume of PBS containing protease inhibitor cocktail was added and the mixture incubated at 20°C for 30 min. Fab fragments were collected by gel filtration using a G50 Sepharose (Sigma, St Louis, MO, USA) column pre-equilibrated in PBS. The absence of any intact IgG in the Fab-containing fractions was confirmed by SDS–polyacrylamide gel electrophoresis. For the double labelling of CCT with the antibodies, 1 µl CCT (2 mg/ml) was incubated with 5 µl of εAD1 Fab fragment and 4 µl CCTδ 8g monoclonal antibody (0.8 mg/ml) for 30 min on ice. Samples were diluted 1:30 with PBS immediately before applying to carbon-coated grids followed by negative staining.

Electron microscopy. For electron microscopy of negatively stained samples, 5 µl aliquots of the CCT–antibody complexes were applied to glow-discharged carbon-coated grids for 1 min and then stained for 1 min with 2% uranyl acetate. Images were recorded at 0°-tilt in a JEOL 1200EX-II electron microscope (JEOL Ltd, Tokyo, Japan) operated at 100 kV and recorded on Kodak SO-163 film at ×60,000 nominal magnification. For cryoelectron microscopy, 5 µl aliquots of a solution containing the purified CCT–antibody complexes were applied to glow-discharged carbon grids for 1 min, blotted for 5 s and frozen rapidly in liquid ethane at −180°C. Images were recorded at 20°-tilt under minimum dose conditions in a JEOL 1200EX-II electron microscope equipped with a Gatan cold stage operated at 100 kV and recorded on Kodak SO-163 film at ×60,000 nominal magnification and between 2.5 and 3.5 µm underfocus.

Image processing, two-dimensional averaging and three-dimensional reconstruction. Micrographs were digitised in a Zeiss SCAI scanner with a sampling window corresponding to 3.5 Å/pixel for all the specimens. For two-dimensional classification and averaging of the negatively stained images, the particles were selected and processed using a free-pattern, maximum-likelihood multi-reference refinement (Scheres *et al*, 2005). As a preliminary three-dimensional reference model, the volume of apo-CCT was used (Llorca *et al*, 2000) with the resolution limited to 40 Å. The EMAN package (Ludtke *et al*, 1999) was used in the first steps of the refinement, until a volume with the two antibodies became evident. The SPIDER software (Frank, 1996) was used in the subsequent iterative refinement procedure. The handedness of the reconstructions was chosen so that it coincided with that of the atomic structure of thermosome from *Thermoplasma acidophilum* (Ditzel *et al*, 1998). The final resolution (20 Å for the CCT–εAD1 complex and 22 Å for the CCT–8g complex) was

estimated with the 0.5 criterion for the Fourier shell correlation coefficient between two independent reconstructions (supplementary Fig S5 online).

Supplementary information is available at *EMBO reports* online (<http://www.emboports.org>)

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