THE EMBO JOURNAL

Manuscript EMBO-2010-76031

The crystal structure of yeast CCT reveals intrinsic asymmetry of eukaryotic cytosolic chaperonins

Carien Dekker, S. Mark Roe, Elizabeth A. McCormack, Fabienne Beuron, Laurence H. Pearl and Keith R. Willison

Corresponding author: Keith R. Willison, Institute of Cancer Research

Review timeline:

Submission date:	17 September 2010
Editorial Decision:	10 November 2010
Revision received:	07 February 2011
Editorial Decision:	09 March 2011
Additional correspondence	09 March 2011
Revision received:	05 April 2011
Editorial Decision:	20 April 2011
Revision received:	27 April 2011
Editorial Decision	10 May 2011
Accepted:	11 May 2011

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 10 November 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. I apologise that it has taken significantly longer than usual to have your manuscript reviewed, but it has been now been evaluated by three referees and I enclose their reports below, as you will see they currently do not support publication in The EMBO Journal.

Upon evaluating the study the referees raise significant concerns with the crystallographic data and the unambiguous order of assignment of subunits. From the reports the referees do not support publication. However, at The EMBO Journal, we have relatively recently started to circulate all the received referee reports with the reviewers for any comments they may have before an editorial decision is made. This is in part an opportunity for people to comment on unfair or potentially biased comments raised by referees. In this case two of the referees feel that you should be given an opportunity to address the issues in a major round of revision. Both referee #2 and #3 agree with the concerns raised by referee #1 regarding the biochemistry in the study and feel that these issues must be addressed. These include further data supporting a role for the described salt bridges including other assays for chaperone function and the use other mutations including Cys crosslinking experiments. There are also a number of issues with the structural data which also need to be resolved, and discussion of previous structural work on CCT is required. From the referees and my point of view it is critical that data provided in the study strongly support the described crystal structure and proposed model to enable it to be used as a basis for future experiments.

© European Molecular Biology Organization

As I mentioned previously there is a consensus that you should be provided with an opportunity to address these points in full. While I appreciate that the referees request a large number of additional experiments, they are critical to the manuscript and will significantly strengthen the main conclusions of the study. I realize that addressing all of the referees' criticisms might require a lot of additional time and effort and be technically challenging, and I would also understand it if you were to rather decide to publish the manuscript rapidly and without any significant changes elsewhere. If you decide to thoroughly revise the manuscript and submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website:
http://www.nature.com/emboj/about/process.html
Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The singular achievement reported here is the preparation of crystals of chaperonin that are (barely) suitable for structure determination by X-ray diffraction. This is a milestone, no doubt. However, there are problems with the biochemical experiments, the clarity of the ms and the conclusions drawn that make this compromise the current submission:

Specifically:

Because the data are of such low resolution, the authors seek to validate their structure solution by biochemical methods. One of these is an experiment where they transform yeast with DNAs expressing either singly mutated CCT6 or CCT6 doubly mutated to recover a putative salt bridge seen in their structure. They conclude that this salt bridge is validated because of the higher rate of false positives seen with the double vs. single mutants. However, this experiment is inadequately described (it is not mentioned at all in Methods). What is a 'false positive' in this case? No Kanr cassette inserted? Something else? How then is kan res. obtained (i.e., how do cell survive selection)? Or is false + something else? Also, would it be possible to do a simple complementation 4 growth assay (i.e, with heterozygous diploid yeast and tetrad analysis?) or (better yet) a functional assay with the various mutant CCT6 chaperonins? All of these would be more straightforward than the described experiment.

Validation is also attempted by mutating the S445 in a putative K113/S445 salt bridge in CCT4 to E (seen in other species) and showing that this mutant supports growth. But this indicates only the the E substitution at this position is viable and provide no direct support for the idea that it forms a salt bridge with K113 (the fact that this is an E in other species but is an S in yeast would seem to be evidence that it does not form a salt bridge with K113:E and S are not only different in charge but also very different in size and shape). Also, why are other substitutions at S445 not tested? If only E or D were viable but K or R or A or T (for example) were not this might support the proposed interaction, but a single substitution is inadequate. Similarly for another putative salt bridge (CCT4 R21/D33) the double swap mutation was created and it is stated that this swap mutation was viable, but it is not stated whether the single mutants were viable (were they even tested?). In the absence of this, the viability of the swap mutant may indicate only that the individual single mutants are viable, and not that a +/- pair is required at this position.

It would have been potentially much more effective if the authors had constructed double cysteine

© European Molecular Biology Organization

substitutions at the sites where they predict amino acids make close approaches in the inter-subunit interactions. The ability to induce disfulfide formation at those sites would have been good evidence for such close approaches.

As a further validation of their structure the authors do a complex dissociation experiment with tagged CCT3 and identify CCT2 and CCT6 as the subunits that remain associated with CCT3 when all other dissociate. This could be evidence that CCT2 and CCT6 are the nearest neighbors of CCT3, but the authors' structure show CCT2 and CCT8 as the nearest neighbors of CCT3 (CCT6 interacts with CCT2). The dissociation assay does not, therefore, appear to be fully reliable as an indicator of nearest neighbor interactions.

The authors state on more than one ocassion that they used a mutant with 'slow ATP kinetics' to faciltate crystallization and achievement of a complex that was (more) homogeneous with respect to nucleotide state. In fact, while they started with ATP and BeF in their crystallization mix, they end up with ADP*BeF in the nucleotide sites. ADP*BeF is a stable ATP ground state analog so the use of a mutant with slower hydrolysis kinetics appears peripheral to their ability to crystallize the chaperonin. They might have done better by starting with ADP*BeF to begin with (and might get better crystals from such set-ups). However, the fact that this mutant also has altered allosteric properties is a problem (and should be mentioned at the outset of the paper) since it could be affecting what is expected to be seen in the structure (i.e., the subunits are not all in the conformation expected for WT in the equivalent nucleotide state due to altered allostery).

Despite the authors' singular achievement in crystallizing this complex, the low resolution of the structure combine with the limited and ambiguous biochemical validation of this structure severely restrict the confidence with which this structure can be used as a basis for an increased understanding of chaperonin mechanism or further experimentation.

Referee #2 (Remarks to the Author):

This paper describes the results of a crystal structure of the yeast CCT together with a number of biochemical experiments with CCT mutants. CCT is made up of two rings and eight distinct but highly similar subunits in each ring. High resolution structure of the CCT is an important endeavor because it provides a baseline for understanding the functional mechanism of this complex protein folding machine.

The strength of this paper is to include a series of biochemical experiments intended to support the suggested models of the eight subunits arrangement in the two rings of CCT. The pitfall of the manuscript is the lack of convincing evidences to support the assignment of the CCT subunits to the crystal structure model. The reasoning of the biochemical observations could be erroneous if the model was not correctly built.

These authors have published a number of papers to suggest the spatial ordering of the CCT subunits based on low resolution electron microscopy with and without folding substrate and antibody labeling. Such spatial ordering was challenged by a recent PNAS paper which was based on a relatively high resolution cryo-EM reconstruction of bovine CCT at ~4 Å resolution. The current paper did not address adequately the differences of their model relative to the recently published model. The manuscript did not present sufficient figures to show if the density supported their model assignment.

I would imagine that a 3.8Å density map should resolve the approximate shapes of some of the large and key side-chains so that the structural data alone can stand on its own in the absence of the other biochemical data to substantiate the subunit arrangement unambiguously. The credibility of the pending paper can be enhanced if they will provide more structural evidences that their subunit assignment is indeed correct so that the readers can establish a trust of the reporting crystal structure and accompanied model.

The followings are the questions that the authors would need to address:

1. The way they determined the arrangement of the CCT eight different subunits in its two rings was

© European Molecular Biology Organization

not convincingly described in this manuscript. The authors mentioned they used the OMIT maps to systematically leaving out two neighboring subunits at a time to improve the difference Fourier maps, but without enough details being described, like why they chose two subunits? Is it too coarse to leave out two subunits at a time, and is the rest 14 subunits enough to compensate such high percentage of missing information since OMIT map usually leaves out ligands or small regions of ambiguous residues only? How many iterations did they use to build the OMIT map? How could they exclude the possibility of model bias from their molecular replacement model? Did they ever use their prior model of subunit ordering within a single ring in their model building process?

2. The authors only showed two small density portions as in Figure 1E-F to demonstrate the quality of their map. However, without showing the more detailed density features in the most sequence diverse apical regions in all the eight subunits, it is hard to judge the resolvability of the map at regions that could allow the investigators to make the subunit assignment to the density unambiguously.

3. The densities of the co-crystallized proteins Plp2 and actin were not clearly resolved in the current crystal structure. The authors claimed the extra density inside one chamber after NCS averaging might belong to actin, and interpreted the CCT substrates and residues interacting with actin. However, the density was not well defined, with only some apparent beta-strand features, based on which the authors indicated this is the small actin subdomain. Have they tried to fit the actin subdomain model to the density? Note that actin subunit has two structurally homologous subdomains (1 and 3). Why do the authors interpret it as subdomain 1 instead of subdomain 3? In addition, extra density is also seen in the other side of the same ring and the opposite ring (Figure S5B), how are these other densities interpreted?

4. In Fig. 3H, the individual 2D raw images are not sufficiently clear to visualize the interaction between CCT-FLAG and anti-FLAG MAb complex. They should perform class-averaging on the images as the EM investigators do. This may resolve such interactions more convincingly. Curiously, why was only one instead of two antibodies bound to each CCT complex?

Referee #3 (Remarks to the Author):

The crystal structure of the CCT oligomer is of major biological importance, since this machine is involved in the folding of key eukaryotic proteins and also appears to play a role in prevention of disease-related aggregation. The overall conformation is very similar to those in the previously published crystal structures of the closed archaeal forms, but the function of CCT also depends on the specific structures of its 8 distinct subunits.

The density inside the cavity attributed to actin is unfortunately not very clear, and there is unexplained density in the opposite ring, as well as on the outside of subunit cct4. Nevertheless, the presence of extra density inside the cavity is interesting.

There is a difference in interpretation of the subunit order within a ring between this paper and the cryo-EM structure published by Frydman and Chiu. The EM structure is at 4.7 A resolution before 2-fold averaging and the present crystal structure is at 3.8 A, so it seems plausible that the present results are more reliable. Nevertheless, this point is not properly dealt with in the manuscript. The statement on p15 that "discussing the differences between the two models would require us to expound on the theory of EM reconstructions at supra-atomic resolution and hence is beyond the scope of this paper" manages to be both pompous and vacuous. In contrast, the authors discuss at some length (pages 5 and 21) EM interpretation in relation to the change in subunit alignment between rings relative to earlier work of the Willison group. The authors should deal with the discrepancy in intra-ring subunit order up front and at least attempt to explain why the results differ, and if/why the present results are more reliable. The EM maps (both 2-fold averaged and unaveraged) are in the EM database, so why cant the authors directly compare the EM density to their coordinates?

Discussion about residue accessibility in the open conformation might be less speculative if the authors make a model of the open state based on the recent archaeal crystal structure (Huo et al,

© European Molecular Biology Organization

Structure 2010).

The writing is sometimes speculative and unclear, e.g. p15 bottom - why should cct6 interface contribute a better understanding of isomer exchange? and p16 2nd par, why should subunit interactions be weakened because cct5 flanks the cct6 interface? The paper would be improved by shortening to remove unnecessary and unclear speculations.

1st Revision - authors' response 07 February 2011

General response to all 3 referees

The major issue that concerns all three reviewers is that the CCT subunit order and inter-ring arrangement in our 3.8A crystal structure of yeast CCT does not agree with the 4A single particle cryo-EM derived structure of bovine testis CCT published by the Chiu and Frydman laboratories in PNAS last year (Cong, Y et al; (2010) 4.0-A resolution cryo-EM structure of the mammalian chaperonin TRiC/CCT reveals its unique subunit arrangement. *Proc Natl Acad Sci U S A*, **107**, 4967-4972). We note that our previous biochemical and electron microscopy data on mouse testis derived CCT has also been challenged in a theoretical and computational analysis of possible CCT ring orders by the communicating editor of this PNAS paper (Kalisman, N and Levitt, M (2009) Insights into the intra-ring subunit order of TRiC/CCT: a structural and evolutionary analysis. *http://eproceedings.worldscinet.com*/9789814295291/preserved-docs/9789814295291_0027.pdf).

Let us first compare the analyses of the intra-ring order of mammalian testis CCT.

The intra-ring order determined by our laboratory (Liou, a.K.F. and Willison,K.R; 1997 ref ibid) is, counter-clockwise in the top ring viewed from the top, using mammalian nomenclature and starting at TCP1/CCTa, is:
AHDQGBZE

The intra-ring order determined by Cong et al (2010), counter-clockwise in the top ring viewed from the top and starting at TCP1/CCTa is:
AZBGQDEH

We note that neither ring-order is compatible with any of the 72 theoretically allowed arrangements of Kalisman and Levitt (2009) based on homology modelling to the solved a/b thermosome structure.

The Cong et al (2010) intra-ring order can be converted to the one of Liou and Willison (1997) by first changing the handedness of the ring:
AHEDQGBZ
and moving subunit E to sit between Z and A
AHDQGBZE

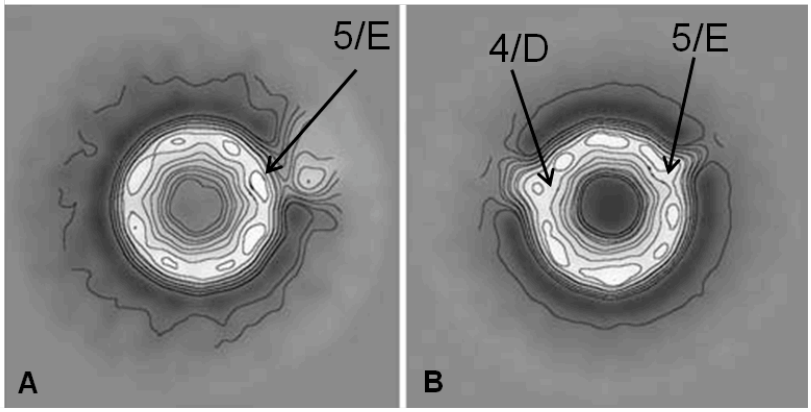
Cong et al (2010) determined a 4.7A structure without imposing any symmetry and found a 2-fold axis between its two rings. A subsequent 2-fold symmetrised map yielded a 4.0A resolution structure that evinced the densities of a large fraction of side chains, loops and insertions and apparently permitted unambiguous identification of all eight individual subunits. New biochemical data was presented which involved formaldehyde cross-linking of TRiC/CCT followed by 2D-PAGE separation. Protein spots corresponding to CCT dimers were excised and the two components identified by MS to provide three pairs of intra-ring subunit associations (NB; the specific locations of the cross-links in the covalent adducts were not mapped). The CCT1/A-CCT7/H and CCT8/Q-CCT3/G pairs agree with Liou and Willison (1997) and the CCT5/E-CCT7/H pair is inconsistent.

Cong et al (2010) did not discuss or take into account any of our 14 various and independent antibody labelling electron microscopy experiments of mouse and bovine testis CCT rings carried out between 1999-2007 (Early work reviewed in Valpuesta et al (2005 ref ibid)). Here below we show an example of antibody labelling of the bovine testis CCT ring taken from Figure 1in Martin-Benito et al (2007 ref ibid).

© European Molecular Biology Organization

Relabelled versions of Figure 1, panels D and E

From Martin-Benito et al; (2007) *EMBO Reports* **8**,252-257



(A,B) Two-dimensional average images from negatively-stained particles. Arrows point to the antibody mass. (A) Two-dimensional average image of the end-on view of the CCT–eAD1 monoclonal antibody complex. (B) Two-dimensional average image of the end-on view of the complex formed between CCT, the intact CCT4/D monoclonal antibody 8g and the Fab fragment of the CCT5/E monoclonal antibody eAD1. The CCT subunits involved with antibody binding (CCT4/D and CCT5/E) are indicated as 4/D and 5/E respectively. eAD1, epsilon apical domain 1; CCT, chaperonin containing TCP-1.

The relative locations of the two subunits in the double antibody-labelled complex in this experiment (panel B) are consistent with Liou and Willison (1997) and inconsistent with Cong et al (2010) in whose model they are adjacently located.

Let us now compare the analyses of the inter-ring order of mammalian testis CCT.

The inter-ring arrangement proposed by Martin-Benito et al (2007) based on low resolution of 3D reconstructions of two bovine testis CCT-MAb complexes by cryo-electron mic,roscopy is:

TOP RING: AHDQGBZE
BOTTOM RING: BGQDHAEZ

The inter-ring arrangement determined by Cong et al (2010) is:

TOP RING: AZBGQDEH
BOTTOM RING: AHEDQGBZ

Cong et al (2010) found a 2-fold axis between the rings in their 4.7A data and also found two cross-linked CCT dimers and used their composition to support their inter-ring arrangement but not the one proposed by Martin-Benito et al (2007); CCT2/B-CCT5/E and CCT8/Q-CCT8/Q.

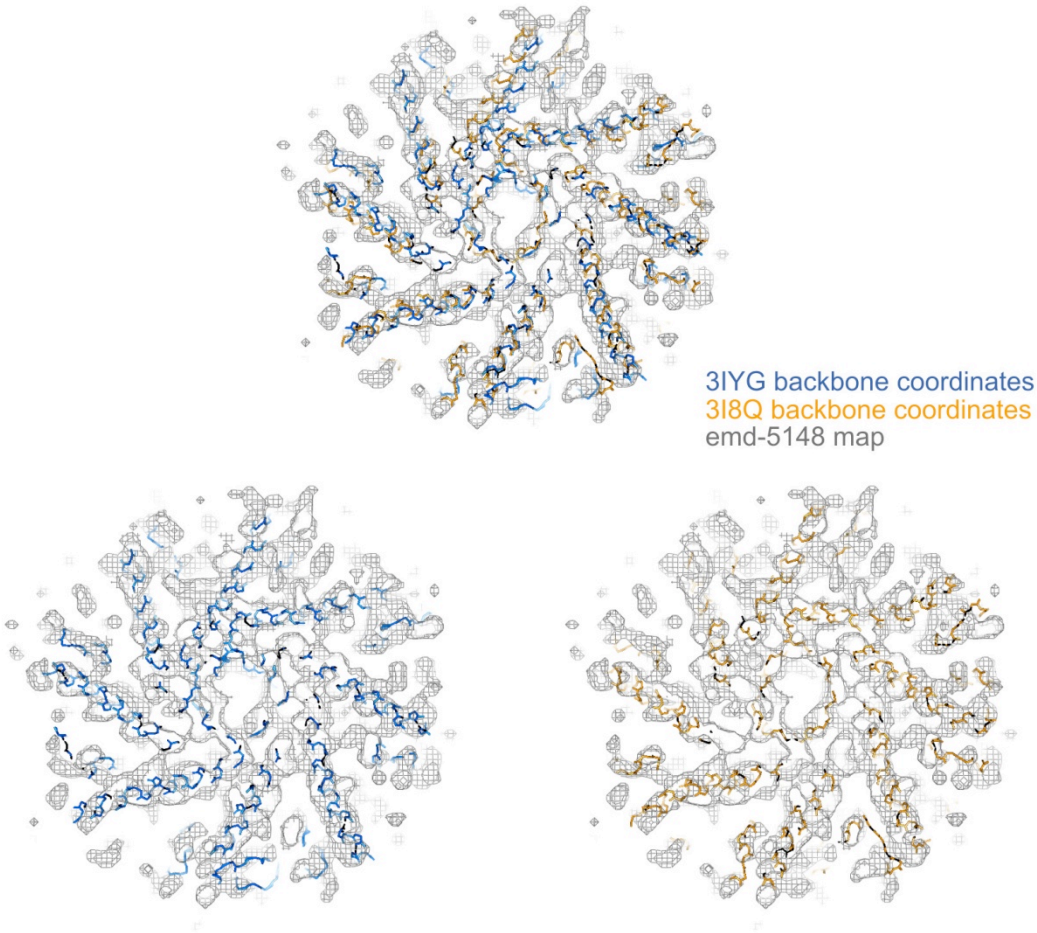
Let us assume that the yeast and mammalian CCT complexes have the same subunit organisation. In our P1 data we found a proper 2-fold axis relating pairs of identical subunits as shown by the self-rotation function (Supplementary Figure S2A,B). This 2-fold axis is consistent with the primary data of Cong et al (2010).
However both the intra- and inter-ring orders in the yeast CCT structure differ from Cong et al (2010) and, continuing to use the mammalian nomenclature rather than yeast subunit numbers for clarity, are:

TOP RING: AHDQGBZE (the same subunit order as Liou and Willison (1997))
BOTTOM RING: GQDHAEZB

© European Molecular Biology Organization

Our yeast CCT inter-ring model differs from the EM model of Martin-Benito et al., (2007) by 45° (one subunit rotation) and as we already discussed in the manuscript the difference between the EM model and the crystal structure can be attributed to the pronounced right-handed twist in each subunit relative to the ring-perpendicular, which was not evident in the low-resolution EM reconstructions. The relative locations of the CCT5/E subunits in the Cong et al (2010) model are 180 ° apart (four subunit rotations) and are thus highly inconsistent with model of Martin-Benito et al (2007). The yeast CCT model is consistent with the CCT2/B-CCT5/E cross-link being derived from an inter-ring pair of subunits. Cong et al (2010) argue that the existence of the CCT8/Q-CCT8/Q dimer makes them an inter-ring pair but in the yeast structure the C-termini of the yeast CCT8 subunits interact with each other across the inter-ring interface and could potentially account for the homomeric, cross-linked dimer.

Response to referee 3 in shown in the figure below: Cong et al (2010) EM density (EMD-5148) compared with their C-a backbone model (3IYG) and with our model (3I8Q)



We prepared this figure in Pymol and it shows the two sets of co-ordinates aligned to the EM map. We judge that the EM map is equally well explained by either set of co-ordinates.
Referee #1 (Remarks to the Author):

The singular achievement reported here is the preparation of crystals of chaperonin that are (barely) suitable for structure determination by X-ray diffraction. This is a milestone, no doubt. However, there are problems with the biochemical experiments, the clarity of the ms and the conclusions drawn that make this compromise the current submission:

Specifically:

Because the data are of such low resolution, the authors seek to validate their structure solution by biochemical methods. One of these is an experiment where they transform yeast with DNAs expressing either singly mutated CCT6 or CCT6 doubly mutated to recover a putative salt bridge

© European Molecular Biology Organization

seen in their structure. They conclude that this salt bridge is validated because of the higher rate of false positives seen with the double vs. single mutants. However, this experiment is inadequately described (it is not mentioned at all in Methods). What is a 'false positive' in this case? No Kanr cassette inserted? Something else? How then is kan res. obtained (i.e., how do cell survive selection)? Or is false + something else? Also, would it be possible to do a simple complementation 4 growth assay (i.e, with heterozygous diploid yeast and tetrad analysis?) or (better yet) a functional assay with the various mutant CCT6 chaperonins? All of these would be more straightforward than the described experiment.

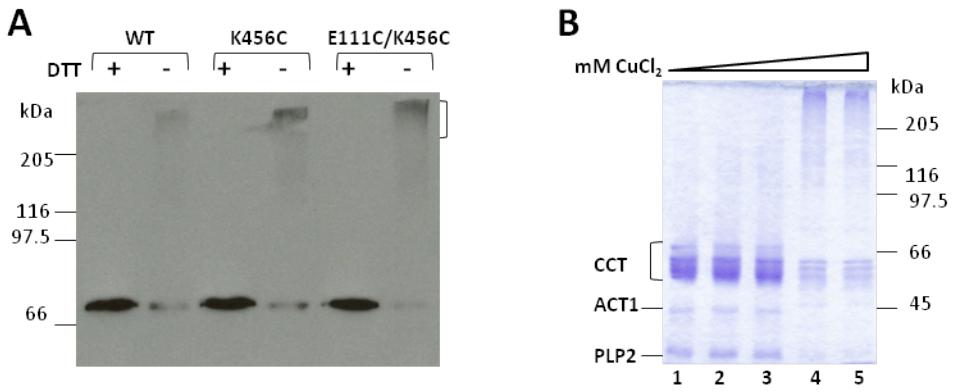
We stated in the manuscript that these experiments were carried out as previously described (Pappenberger et al., 2006 ref ibid) but we appreciate that the screening strategy was poorly described and confusing. We have rewritten the description as well as adding a section to the Materials and Methods in the revised manuscript. When one transforms haploid yeast with linear fragments of genes with a Kan^r cassette at the 5' or 3' end of the fragment one obtains a large background from non-homologous recombination events which result in G418 resistant colonies. One also obtains homologous recombination events which don't occur through both ends of the fragment so that the Kan^r cassette is inserted but not upstream or downstream sequences containing the tag or the desired mutation. Our strategy for counting the events is to PCR-screen across the internal purification tag insertion using primers from either end of the target gene and this allows us to discriminate between non-homologous events and homologous events in the target gene because the internal purification tag sequence adds 165 bps to the DNA product of the PCR fragment. The PCR products of homologous events are subjected to DNA sequencing to verify the presence or not of the desired mutation(s). This is a very straightforward approach for essential genes such as the 8 CCT genes. We don't believe that the suggestion of performing mutational analysis in diploid yeast is more straightforward because of the haplo-insufficient expression behaviour of the CCT genes (Deutschbauer, A.M., et al (2005) Mechanisms of haploinsufficiency revealed by genome-wide profiling in yeast. Genetics **169** 1915-1925) and also the potentially toxic consequences of assembling the heteromeric CCT complex from mixtures of wild-type and lethal subunits. We have used the same haploid cell transformation approach to introduce mutations into the ATP sites of all eight CCT subunits (Amit et al., 2010 ref ibid). In this study we could not obtain the D91E mutation in the Cct4 gene and attributed this to lethality rather than a technical issue to do with the transformation strategy. We proved that the result was not due to technical problems by introducing a silent mutation at position 91 codon in CCT4 which resulted in a viable strain, thereby indicating that the mutation D91E in CCT4 was responsible for the loss of viability.

Validation is also attempted by mutating the S445 in a putative K113/S445 salt bridge in CCT4 to E (seen in other species) and showing that this mutant supports growth. But this indicates only the the E substitution at this position is viable and provide no direct support for the idea that it forms a salt bridge with K113 (the fact that this is an E in other species but is an S in yeast would seem to be evidence that it does not form a salt bridge with K113: E and S are not only different in charge but also very different in size and shape). Also, why are other substitutions at S445 not tested? If only E or D were viable but K or R or A or T (for example) were not this might support the proposed interaction, but a single substitution is inadequate. Similarly for another putative salt bridge (CCT4 R21/D33) the double swap mutation was created and it is stated that this swap mutation was viable, but it is not stated whether the single mutants were viable (were they even tested?). In the absence of this, the viability of the swap mutant may indicate only that the individual single mutants are viable, and not that a +/- pair is required at this position.

It would have been potentially much more effective if the authors had constructed double cysteine substitutions at the sites where they predict amino acids make close approaches in the inter-subunit interactions. The ability to induce disfulfide formation at those sites would have been good evidence for such close approaches.

We carried out this type of analysis for the CCT6-CCT6 salt bridge interface specifically for this refereee. We constructed haploid yeast strains containing the single mutant E111C and the double mutant E111C/K456C along with the CBP-tag inserted into CCT6; the same protocol used for the salt-bridge charge swap in the manuscript (Figure 2 A-E). We note that the E111C strain is viable, in contrast to the E111K strain, showing that the interface is able to tolerate a neutral charge opposite one of the salt bridge positions. We followed the biochemical protocol of Velichutina et al; (2004) who performed disulphide engineering on the a-subunits rings of the yeast 20S proteasome to

confirm juxtaposition of neighbouring subunits, such as pre8a2-S-S- Pre9a3, from the crystal stucture model. Velichutina et al; (2004) use $CuCl_2$-induced cross linking followed by analysis on SDS-PAGE with and without dithiothreitol (DTT) in the gel loading sample buffer. Cal-resin purified CCT-complexes were treated with 0.2mM $CuCl_2$ and analysed on denaturing SDS-PAGE gels in the presence and absence of DTT. Panel A in the figure below shows 10% SDS-PAGE Western blotting analysis with anti-CCT6 subunit antibody of wild type CCT-6CBP (lanes WT), K456C mutant CCT-6CBP (lanes K456C) and E111C/K456C double mutant CCT-6CBP (lanes E111C/K456C)



Unfortunately treatment of CCT with $CuCl_2$ causes extensive aggregation of wild type CCT-6CBP and both the mutant CCTs with most of the signal shifting to the interface between the stacking and resolving gels (bracket on rhs of panel A). We checked to establish the effect of $CuCl_2$ treatment on preassembled CCT-ACT1-PLP2 complexes and found similar behaviour with an abrupt transition to ill-defined aggregated states at around 0.5mM $CuCl_2$ (panel B, lane 4).

We have previously shown the requirement for DTT or TCEP in all buffers when working with folding competent yeast CCT; the presence of either agent is essential for efficient substrate release (McCormack et al; 2009). Each yeast CCT subunit has between 5 and 11 cysteine residues bringing the total number per 16-mer CCT complex to 122. We performed experiments to obtain an indication of the number of cysteines in CCT which have free sulphydryls that contribute to this DTT-dependent behaviour through the use of a blocking agent N-ethylmaleimide (NEM) in conjunction with Ellman's reagent (DTNB). What we discovered is that CCT is highly sensitive to NEM. CCT was incubated with increasing concentrations of NEM for 10min and NEM was then removed (using zeba-columns) and DTNB was added (DTNB was calibrated against cysteine and free sulphydryls were calculated using : [-SH] = volume (ml) * (A_{412}sample - A_{412}blank) / (De_{412} * 1cm). The De_{412} was determined to be 10,420 M^{-1cm-1}. The results showed a saturation effect; all readily accessible cysteines had been blocked at an NEM concentration of 1mM. Gel analysis showed that NEM actively destabilises CCT and induces extensive aggregation of CCT complexes. We believe that oxidised cysteine residues may interfere with certain movements or conformational changes in CCT. We also draw your attention to our previous discovery of a disulphide bond in the apical domain of the mouse CCTg subunit in our 2.2A X-ray structure of this domain (Pappenberger et al 2002).

In summary we found that cys-cys disulphide engineering, followed by cross-linking, was not applicable to yeast CCT subunit-subunit associations. However we investigated other approaches and found that we could use diazerine cross-linking reagents to cross-link actin and PLP2 to CCT subunits and we describe these experiments in Figures 6 and 7 of the new manuscript. Together they provide strong support for the actin density assignment and the CCT subunit assignments in the actin binding half of the ring (see revised Discussion section).

McCormack, E.A., Altschuler, G.M., Dekker, C., Filmore, H. and Willison, K.R. (2009) Yeast phosducin-like protein 2 acts as a stimulatory co-factor for the folding of actin by the chaperonin CCT via a ternary complex. *Journal of Molecular Biology*, **391**, 192-206.
Pappenberger, G., Wilsher, J.A., Roe, S.M., Counsell, D.J., Willison, K.R. and Pearl, L.H. (2002) Crystal structure of the CCT gamma apical domain: Implications for substrate binding to the eukaryotic cytosolic chaperonin. *Journal of Molecular Biology*, **318**, 1367-1379.

© European Molecular Biology Organization

Velichutina, I., Connerly, P.L., Arendt, C.S., Li, X., and Hochstrasser, M. (2004) Plasticity in eukaryotic 20S proteasome ring assembly revealed by a subunit deletion in yeast. *EMBO.J* **23**, 500-510.

As a further validation of their structure the authors do a complex dissociation experiment with tagged CCT3 and identify CCT2 and CCT6 as the subunits that remain associated with CCT3 when all other dissociate. This could be evidence that CCT2 and CCT6 are the nearest neighbors of CCT3, but the authors' structure show CCT2 and CCT8 as the nearest neighbors of CCT3 (CCT6 interacts with CCT2). The dissociation assay does not, therefore, appear to be fully reliable as an indicator of nearest neighbor interactions.

Why should the tagged subunit necessarily be the one in the middle? We argue that the strength of the neighboring subunit interactions between *Cct5* and *Cct6* is weakened within the ring, due to the 3-stranded b-sheet rather than 4-stranded b-sheet interaction (Figure 3D). Also our previous work with testis CCT found that the CCT8/q subunit was always monomeric and did not form micro-complexes with other subunits (Liou and Willison 1997 ref ibid).

*CCT8-**CCT3-CCT2-CCT6**-CCT5*

Therefore from this segment of the ring the ***CCT3-CCT2-CCT6*** trimer seems stable. We note that this trimer is present in the Cong et al (2010) ring model.

*The authors state on more than one ocassion that they used a mutant with 'slow ATP kinetics' to faciltate crystallization and achievement of a complex that was (more) homogeneous with respect to nucleotide state. In fact, while they started with ATP and BeF in their crystallization mix, they end up with ADP*BeF in the nucleotide sites. ADP*BeF is a stable ATP ground state analog so the use of a mutant with slower hydrolysis kinetics appears peripheral to their ability to crystallize the chaperonin. They might have done better by starting with ADP*BeF to begin with (and might get better crystals from such set-ups). However, the fact that this mutant also has altered allosteric properties is a problem (and should be mentioned at the outset of the paper) since it could be affecting what is expected to be seen in the structure (i.e., the subunits are not all in the conformation expected for WT in the equivalent nucleotide state due to altered allostery).*

We tried many variations of analogues and this was the only condition that gave good diffraction. The complex was crystallised in hanging drop in the presence of ATP and Beryllium Fluoride, which was added as $BeSO_4$ and KF. Equilibration buffer contained 100 mM Hepes pH 7.6, 50 mM $MgCl_2$, 300 mM Na_2SeO_4, 6% PEG8k,1.0 mM TCEP, and 20% glycerol. We used these same conditions with ADP but did not obtain useable crystals.

As we already discussed in the manuscript we expect that not all the subunits are in the conformation expected for wild type. Similar problems have been found for closed thermosome crystal structures and similar arguments pertain (Shomura, Y et al., 2004 ref ibid). We have now stated this at the outset of the manuscript.

Despite the authors' singular achievement in crystallizing this complex, the low resolution of the structure combine with the limited and ambiguous biochemical validation of this structure severely restrict the confidence with which this structure can be used as a basis for an increased understanding of chaperonin mechanism or further experimentation.

This is quite a harsh final comment, especially without further indication by the referee why the biochemical validation is limited or ambiguous. In this revised manuscript we have added substantial additional experiments that are all consistent with our model, providing us with the confidence to publish our data to allow others to work with and test our model.

© European Molecular Biology Organization

Referee #2 (Remarks to the Author):

This paper describes the results of a crystal structure of the yeast CCT together with a number of biochemical experiments with CCT mutants. CCT is made up of two rings and eight distinct but highly similar subunits in each ring. High resolution structure of the CCT is an important endeavour because it provides a baseline for understanding the functional mechanism of this complex protein folding machine.

The strength of this paper is to include a series of biochemical experiments intended to support the suggested models of the eight subunits arrangement in the two rings of CCT. The pitfall of the manuscript is the lack of convincing evidences to support the assignment of the CCT subunits to the crystal structure model. The reasoning of the biochemical observations could be erroneous if the model was not correctly built.

These authors have published a number of papers to suggest the spatial ordering of the CCT subunits based on low resolution electron microscopy with and without folding substrate and antibody labelling. Such spatial ordering was challenged by a recent PNAS paper which was based on a relatively high resolution cryo-EM reconstruction of bovine CCT at ~4 Å resolution. The current paper did not address adequately the differences of their model relative to the recently published model. The manuscript did not present sufficient figures to show if the density supported their model assignment.

I would imagine that a 3.8Å density map should resolve the approximate shapes of some of the large and key side-chains so that the structural data alone can stand on its own in the absence of the other biochemical data to substantiate the subunit arrangement unambiguously. The credibility of the pending paper can be enhanced if they will provide more structural evidences that their subunit assignment is indeed correct so that the readers can establish a trust of the reporting crystal structure and accompanied model.

The followings are the questions that the authors would need to address:

1. The way they determined the arrangement of the CCT eight different subunits in its two rings was not convincingly described in this manuscript. The authors mentioned they used the OMIT maps to systematically leaving out two neighboring subunits at a time to improve the difference Fourier maps, but without enough details being described, like why they chose two subunits? Is it too coarse to leave out two subunits at a time, and is the rest 14 subunits enough to compensate such high percentage of missing information since OMIT map usually leaves out ligands or small regions of ambiguous residues only? How many iterations did they use to build the OMIT map? How could they exclude the possibility of model bias from their molecular replacement model? Did they ever use their prior model of subunit ordering within a single ring in their model building process?

The rationale behind leaving out two neighbouring subunits from one copy of the two CCT complexes only (2/32 subunits) was that it equates to about 6% of the model whereas in standard OMIT maps one typically leaves out 5% of the model (or 20 OMIT regions that tile to cover the asymmetric unit, see Terwilliger et al., (2008) *Acta Cryst* D64, 515-524). The molecular replacement model used was an all alpha-thermosome model. One can never exclude model bias entirely but the appearance of unique and asymmetrically distributed density features could only have come from the CCT data. We did not use our prior Liou and Willison model at any stage in the subunit assignment process.

2. The authors only showed two small density portions as in Figure 1E-F to demonstrate the quality of their map. However, without showing the more detailed density features in the most sequence diverse apical regions in all the eight subunits, it is hard to judge the resolvability of the map at regions that could allow the investigators to make the subunit assignment to the density unambiguously.

Figures 1 E and F were included to illustrate the density of a stretch of sequence with low homology. The subunit assignments were based on the equatorial domains only. Please note that the sequence homology of CCT subunits is only of the order of 35% identity in pair-wise comparisons

© European Molecular Biology Organization

and there is substantial divergence between equatorial domains. We have included more density figures in the Supplementary data (Supplementary Fig1 F,G,H) to give a fair indication of the 3.8A data.

3. The densities of the co-crystallized proteins Plp2 and actin were not clearly resolved in the current crystal structure. The authors claimed the extra density inside one chamber after NCS averaging might belong to actin, and interpreted the CCT substrates and residues interacting with actin. However, the density was not well defined, with only some apparent beta-strand features, based on which the authors indicated this is the small actin subdomain. Have they tried to fit the actin subdomain model to the density? Note that actin subunit has two structurally homologous subdomains (1 and 3). Why do the authors interpret it as subdomain 1 instead of subdomain 3?

In addition, extra density is also seen in the other side of the same ring and the opposite ring (Figure S5B), how are these other densities interpreted?

We protected the density in the cavity from solvent flattening. It is difficult to see this in 2D representation but the density in the opposite ring is far less extensive and is not continuous whereas the density in the actin containing ring is continuous.

4. In Fig. 3H, the individual 2D raw images are not sufficiently clear to visualize the interaction between CCT-FLAG and anti-FLAG MAb complex. They should perform class-averaging on the images as the EM investigators do. This may resolve such interactions more convincingly. Curiously, why was only one instead of two antibodies bound to each CCT complex?

The disposition of the anti-FLAG MAb relative to the CCT complex is highly heterogeneous on the EM grids and class-averaging will remove the signal entirely. We do find a very few complexes with two antibodies bound but as we explained, although we purified the CCT-MAb complexes on sucrose gradients we conducted the analysis at low molar ratio of CCT to antibody (1:0.5) and an available site ratio of 2:0.5 in order to avoid the possibility of any free MAb confounding the analysis. It is also possibility that steric hindrance is an issue since the two Cct5 subunits are closely located on either side of the Cct6 pair (see Figure 1C).

Referee #3 (Remarks to the Author):

The crystal structure of the CCT oligomer is of major biological importance, since this machine is involved in the folding of key eukaryotic proteins and also appears to play a role in prevention of disease-related aggregation. The overall conformation is very similar to those in the previously published crystal structures of the closed archaeal forms, but the function of CCT also depends on the specific structures of its 8 distinct subunits.

The density inside the cavity attributed to actin is unfortunately not very clear, and there is unexplained density in the opposite ring, as well as on the outside of subunit cct4. Nevertheless, the presence of extra density inside the cavity is interesting.

Our new cross-linking results strongly support our attribution of the density inside the cavity to actin (Figures 6 and 7 in the revised manuscript).
As we replied to referee 2 point 3; it is difficult to see this in 2D representation but the density in the opposite ring is far less extensive and is not continuous whereas the density in the actin containing ring is continuous.

There is a difference in interpretation of the subunit order within a ring between this paper and the cryo-EM structure published by Frydman and Chiu. The EM structure is at 4.7 A resolution before 2-fold averaging and the present crystal structure is at 3.8 A, so it seems plausible that the present results are more reliable. Nevertheless, this point is not properly dealt with in the manuscript. The statement on p15 that "discussing the differences between the two models would require us to expound on the theory of EM reconstructions at supra-atomic resolution and hence is beyond the scope of this paper" manages to be both pompous and vacuous. In contrast, the authors discuss at

© European Molecular Biology Organization

some length (pages 5 and 21) EM interpretation in relation to the change in subunit alignment between rings relative to earlier work of the Willison group. The authors should deal with the discrepancy in intra-ring subunit order up front and at least attempt to explain why the results differ, and if/w
hy the present results are more reliable. The EM maps (both 2-fold averaged and unaveraged) are in the EM database, so why cant the authors directly compare the EM density to their coordinates?

We discuss this at the beginning of this rebuttal. We show a figure of direct comparison of the EM density to our structure on page 3 of this response. We do not have the space to include the discussion in the manuscript due to lack of space.

Discussion about residue accessibility in the open conformation might be less speculative if the authors make a model of the open state based on the recent archaeal crystal structure (Huo et al, Structure 2010).

We are reluctant to build models based upon archaeal structures. Each CCT subunit has unique properties.

The writing is sometimes speculative and unclear, e.g. p15 bottom - why should cct6 interface contribute a better understanding of isomer exchange? and p16 2nd par, why should subunit interactions be weakened because cct5 flanks the cct6 interface? The paper would be improved by shortening to remove unnecessary and unclear speculations.

We think the Cct5-Cct6 subunit interactions are weakened due to the fact that the interface is only via a 3-stranded b-sheet and not a 4-stranded b-sheet as found for all the other interaction surfaces; this is because the N-terminus of subunit Cct5 does not run from the inside of the cavity, like the N-termini of all other subunits, but threads in from the outside of the CCT complex (as we have explained in the section on page 9 of the manuscript - *Unique position of the Cct5 N-terminus*).

We have removed some of the unnecessary and unclear speculations in the discussion (p16-18) to improve the manuscript and also to make space for the new cross-linking data (Figures 6 and 7 of the revised manuscript).

2nd Editorial Decision 09 March 2011

Your revised manuscript has been reviewed once more by two of the original referees. They find that you have not satisfactorily addressed their earlier concerns, this usually results in the study being rejected post review. However, after discussing the comments with the referees they would like to offer you one more opportunity to address the remaining concerns. However, we do need to be clear that if this cannot be resolved in the next round final round of revision we will not be able to offer publication in The EMBO Journal.

The final requests by referee #1 should be addressed and satisfactory responses provided to the comments from referee #3, which after further discussion should include some way of briefly dealing with the discrepancy with the Chiu study. Ideally with a figure to clearly demonstrate critical regions where the respective density maps should discriminate the two models.

When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

© European Molecular Biology Organization

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

My original criticisms of this manuscript were of the biochemical sections and, as the other reviewers have adequately focussed on the x-ray study, I will continue to address the biochemical studies.

1. Cross-linking and salt bridge swaps to validate inter-subunit interactions:

i. In response to criticism that salt-bridge swap experiments did not adequately support inter-subunit interaction model, the reviewers tried engineering a disulfide at E111/K456. Unfortunately, the WT and single cys and double cys mutants all seemed to aggregate upon addition of the oxidizing agent (CuCl2) used to induce disulfide formation so this experiment failed. It is possible that this experiment would have failed no matter how it was attempted. However, it is unfortunate that only CuCl2 seems to have been tried as the oxidizing agent. Copper2 is known to be a powerful protein aggregant (see, for example, Cell Mol Life Sci. 2004 Apr;61(7-8):982-91.Investigation of the effects of copper ions on protein aggregation using a model system). It is possible that the experiment might have worked with other oxidants (HEDS, oxidized glutathione) but we don't know. In any case, this particular experiment failed so it provided no support for the proposed inter-subunit interaction. The authors do note that the E111C substitution was viable while E111K was not (I assume also E111C/K456C was viable). The authors suggest this supports their E111-K456 salt bridge but both the E11C and E111C-K456C mutations should disrupt or at least weaken this salt bridge so the viability of the cys subtituted enyzmes is problematic in terms of accepting that the E111-K456 salt bridge is present and essential for viability. Further, the viability of these cys subsitutions undermines the statement that: "The successful introduction of charge-swap mutations at specific sites in line with the current model is all the more compelling since CCT, as an essential complex in vivo in yeast, hardly tolerates even mild mutations at conserved sites at all (Amit et al., 2010)". In fact, the cys subsitutions seem well-tolerated.

ii. Regarding the other swap mutations the original criticisms persist: the putative K113-S445 was tested only by changing S445 to E and showing viability, but an E or D is seen at the 445 position in other (non cervesiae) CCTs so all this shows is that E is a viable subsititution here. The fact that 445 can be an S, D, or E in different CCTs would argue that it does NOT make a salt bridge to conserved K113 since it is unclear how residues as different in size and charge as S and E could both make a similar salt bridge. Similarly, the viability of the R21D/D33R mutation--in the absence of further mutations or tests of the viability of the single mutants--does not, in itself, support the salt bridge hypothesis: perhaps the single mutants would also be viable.

2. The authors also use a complex dissociation experiment with tagged CCT3 to attempt to identify the nearest neighbors of this subunit. They explicitly state that this assay is intended to identify NEAREST NEIGHBORS: "The dependency of yeast CCT complex stability on glycerol allows for an alternative way of identifying nearest neighbours within the complex, making use of the purification tags located on the outside of the CCT complex but without interfering with subunit interactions as would be the case when using crosslinkers" In their structure the nearest neighbors of CCT3 are CCT2 and CCT8 (which sit on either side of CCT3) but the assay picks up CCT2 and CCT6 in the sub-complex with CCT3. I am perfectly satisfied with the authors' explanation that this may be because the CCT3 interacton with CCT8 is weak but the problem is that the authors put this assay forward as a way to identify the NEAREST NEIGHBORS of CCT3 and therefore to validate their structure. My criticism that this assay is not a reliable indicator of nearest neighbors remains. I do not underatand why the authors argue this point. In their rebuttal they ask:
" Why should the tagged subunit necessarily be the one in the middle?"; to which may answer is: "Because the ones on either side are its nearest neighbors and you said that the assay was intended to identify its nearest neighbors. If the assay doesn't do that then it is an unreliable indicator of the

© European Molecular Biology Organization

nearest neighbors".

The authors do have new experiments showing actin and PL2 cross-linking to CCT subunits but these do not address the criticisms made above and, oddly, these new experiments (in fig. 7) are not even incorporated into the "results" section of the ms but simply described in the "discussion". The authors wonder why I characterize the biochemical data as "limited and ambiguous" and want me to be more explicit about this, but I believe I was quite explicit about this both in the original review and, again, in my comments above.

I am however sympathetic to the challenges of working with this unstable and essential protein complex and very impressed by their success in obtaining even a low res structure. If the other reviewers are satisfied with the structural studies I would be supportive of publication if: 1. The data on the salt-bridge swap mutants are simple removed. These are too ambiguous to be useful and simply dilute this study and confuse the manuscript. 2. The caveats of the complex dissociation assay--that it does not merely report nearest neighbors but may miss some of the nearest neighbors as it is also affected by interaction strength--is laid out more clearly. 3. The new data in fig. 7 needs to be incorporated into the Results section. 4.These changes allow a tighter, clearer manuscript to be written.

Referee #3 (Remarks to the Author):

Does the interpretation of the actin cross linking results depend on the model of how the actin domains are arranged in the chaperonin cavity? This model is based on very low resolution EM data and is quite speculative.

The comparison of the Frydman/Chiu map with the two atomic models is not very helpful. It shows a single section through the apical domains, rather than focussing on regions where the subunit order is expected to make a difference. Is there any difference in cross correlation between the two sets of atomic coordinates and the Chiu map? The authors' refusal to discuss this issue in the paper does not help to resolve the confusion around the key point of subunit order.

Additional correspondence (author) 09 March 2011

Thanks for your support with this manuscript and for giving us another chance to satisfy the referees.

I will revise the manuscript according to referee #1 by removing the yeast salt bridge mutant data, clarifying the complex dissociation experiment and moving our new actin cross-linking data to the results section. For referee #3 we will briefly discuss the Chiu model in the main body of the paper and will include a Figure showing some comparisons of density maps.

2nd Revision - authors' response 05 April 2011

Response to referees

Referee #1 (Remarks to the Author):

My original criticisms of this manuscript were of the biochemical sections and, as the other reviewers have adequately focussed on the x-ray study, I will continue to address the biochemical studies.

1. Cross-linking and salt bridge swaps to validate inter-subunit interactions:

i. In response to criticism that salt-bridge swap experiments did not adequately support inter-subunit interaction model, the reviewers tried engineering a disulfide at E111/K456. Unfortunately,

© European Molecular Biology Organization

the WT and single cys and double cys mutants all seemed to aggregate upon addition of the oxidizing agent (CuCl2) used to induce disulfide formation so this experiment failed. It is possible that this experiment would have failed no matter how it was attempted. However, it is unfortunate that only CuCl2 seems to have been tried as the oxidizing agent. Copper2 is known to be a powerful protein aggregant (see, for example, Cell Mol Life Sci. 2004 Apr;61(7-8):982-91.Investigation of the effects of copper ions on protein aggregation using a model system). It is possible that the experiment might have worked with other oxidants (HEDS, oxidized glutathione) but we don't know. In any case, this particular experiment failed so it provided no support for the proposed inter-subunit interaction. The authors do note that the E111C substitution was viable while E111K was not (I assume also E111C/K456C was viable). The authors suggest this supports their E111-K456 salt bridge but both the E11C and E111C-K456C mutations should disrupt or at least weaken this salt bridge so the viability of the cys subtituted enyzmes is problematic in terms of accepting that the E111-K456 salt bridge is present and essential for viability. Further, the viability of these cys subsitutions undermines the statement that: "The successful introduction of charge-swap mutations at specific sites in line with the current model is all the more compelling since CCT, as an essential complex in vivo in yeast, hardly tolerates even mild mutations at conserved sites at all (Amit et al., 2010)". In fact, the cys subsitutions seem well-tolerated.

ii. Regarding the other swap mutations the original criticisms persist: the putative K113-S445 was tested only by changing S445 to E and showing viability, but an E or D is seen at the 445 position in other (non cervesiae) CCTs so all this shows is that E is a viable subsititution here. The fact that 445 can be an S, D, or E in different CCTs would argue that it does NOT make a salt bridge to conserved K113 since it is unclear how residues as different in size and charge as S and E could both make a similar salt bridge. Similarly, the viability of the R21D/D33R mutation--in the absence of further mutations or tests of the viability of the single mutants--does not, in itself, support the salt bridge hypothesis: perhaps the single mutants would also be viable.

2. The authors also use a complex dissociation experiment with tagged CCT3 to attempt to identify the nearest neighbors of this subunit. They explicitly state that this assay is intended to identify NEAREST NEIGHBORS: "The dependency of yeast CCT complex stability on glycerol allows for an alternative way of identifying nearest neighbours within the complex, making use of the purification tags located on the outside of the CCT complex but without interfering with subunit interactions as would be the case when using crosslinkers" In their structure the nearest neighbors of CCT3 are CCT2 and CCT8 (which sit on either side of CCT3) but the assay picks up CCT2 and CCT6 in the sub-complex with CCT3. I am perfectly satisfied with the authors' explanation that this may be because the CCT3 interacton with CCT8 is weak but the problem is that the authors put this assay forward as a way to identify the NEAREST NEIGHBORS of CCT3 and therefore to validate their structure. My criticism that this assay is not a reliable indicator of nearest neighbors remains. I do not underatand why the authors argue this point. In their rebuttal they ask:
" Why should the tagged subunit necessarily be the one in the middle?"; to which may answer is: "Because the ones on either side are its nearest neighbors and you said that the assay was intended to identify its nearest neighbors. If the assay doesn't do that then it is an unreliable indicator of the nearest neighbors".

The authors do have new experiments showing actin and PL2 cross-linking to CCT subunits but these do not address the criticisms made above and, oddly, these new experiments (in fig. 7) are not even incorporated into the "results" section of the ms but simply described in the "discussion". The authors wonder why I characterize the biochemical data as "limited and ambiguous" and want me to be more explicit about this, but I believe I was quite explicit about this both in the original review and, again, in my comments above.

I am however sympathetic to the challenges of working with this unstable and essential protein complex and very impressed by their success in obtaining even a low res structure. If the other reviewers are satisfied with the structural studies I would be supportive of publication if: 1. The data on the salt-bridge swap mutants are simple removed. These are too ambiguous to be useful and simply dilute this study and confuse the manuscript. 2. The caveats of the complex dissociation assay--that it does not merely report nearest neighbors but may miss some of the nearest neighbors as it is also affected by interaction strength--is laid out more clearly. 3. The new data in fig. 7 needs to be incorporated into the Results section. 4.These changes allow a tighter, clearer manuscript to be written.

© European Molecular Biology Organization

1. We have removed the salt-bridge swap mutant data and Figure 2 completely.

2. We have laid out the caveats of the complex dissociation assay and revised the description of the experiment to make it clearer.

3. We have incorporated the new data in Figure 7 into the Results section and have written a new section in the Materials and Methods describing the CCT-actin-PLP2 cross-linking experiments (now Figures 5 and 6 in the revised manuscript).

Referee #3 (Remarks to the Author):

Does the interpretation of the actin cross linking results depend on the model of how the actin domains are arranged in the chaperonin cavity? This model is based on very low resolution EM data and is quite speculative.

No, the interpretation is based upon the new cross-linking data only.

The comparison of the Frydman/Chiu map with the two atomic models is not very helpful. It shows a single section through the apical domains, rather than focussing on regions where the subunit order is expected to make a difference.

Is there any difference in cross correlation between the two sets of atomic coordinates and the Chiu map?

Unfortunately Frydman/Chiu did not deposit the atomic co-ordinates of their model; just a poly-alanine Ca-trace (PDB entry 3IYG). The Frydman/Chiu 4.0Ang map (emd_5148) deposited in the EMD also has a different origin to the deposited PDB model, hence the model had to be placed by hand. These omissions have made the models harder to compare.

The authors' refusal to discuss this issue in the paper does not help to resolve the confusion around the key point of subunit order.

We have performed a direct comparison of the two structures in the region that Frydman/Chiu use to discriminate the Cct5 subunit in their structure and present this analysis in a figure (new Figure 8) and accompanying discussion subsection (*Comparison of yeast X-ray structure with previous cryo-EM models*).

3rd Editorial Decision 20 April 2011

Your manuscript has been reviewed by one of the original referees who is really expending substantial effort to make suggestions that would make the manuscript suitable for The EMBO Journal. THe final comments are listed below with changes that are required for the manuscript, this mainly involves removing references to the manual docking. PLease incorporate these final corrections.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

© European Molecular Biology Organization

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #3 (Remarks to the Author):

comments to authors

There is a silly technical problem with the comparison that was added, at my request, with the Chiu structure. The Chiu coordinates were not aligned with the map, and the authors say they had to place it by hand. That is not good enough in a contentious comparison like this. It is perfectly feasible to do the alignment with Chimera or other software, but in the meantime this seems to have been fixed anyway - I just downloaded the files and they are now aligned (I think the map file was corrected). This should be fixed and the reference to manual placement removed.

Moreover, the comparison in the new Fig 8 is extremely hard to follow - can this be explained more clearly, eg with a little ring diagram with the subunit orders? It would seem have been more straightforward to compare 2 views of 4.7 A EM map with the alternative coordinates (ie from EM and from crystal structure) docked in. Nonetheless, the figure makes the point that the crystallography map is of far higher quality than the EM ones, which dont look as if they could discriminate the subunits.

3rd Revision - authors' response 27 April 2011

Response to referees

Referee #3 (Remarks to the Author):

comments to authors

There is a silly technical problem with the comparison that was added, at my request, with the Chiu structure. The Chiu coordinates were not aligned with the map, and the authors say they had to place it by hand. That is not good enough in a contentious comparison like this. It is perfectly feasible to do the alignment with Chimera or other software, but in the meantime this seems to have been fixed anyway - I just downloaded the files and they are now aligned (I think the map file was corrected). This should be fixed and the reference to manual placement removed.

We thank this referee for notifying us that the maps had been updated. Please note that we have regenerated the relevant panels in Figure 8 (new panels F, G and H) with the corrected maps and we used the contour levels recommended as indicated in the author's updated EMDB entry. We are now confident that we are showing what is publicly accessible.

Moreover, the comparison in the new Fig 8 is extremely hard to follow - can this be explained more clearly, eg with a little ring diagram with the subunit orders? It would seem have been more straightforward to compare 2 views of 4.7 A EM map with the alternative coordinates (ie from EM and from crystal structure) docked in. Nonetheless, the figure makes the point that the crystallography map is of far higher quality than the EM ones, which dont look as if they could discriminate the subunits.

We have added two extra panels to our revised Figure 8 which show ring diagrams of the yeast X-ray and bovine EM subunit orders (panel A) and highlight the position of the Cct5 subunit with respect to the two-fold axis (indicated by a blue line). We also moved the sequence alignment panel up to panel B (was panel G in previous version) for clarity. We thank the referee for these suggestions and we hope that the revised Figure 8 and accompanying explanation are less hard to follow.

© European Molecular Biology Organization

4th Editorial Decision 10 May 2011

Your revised manuscript has been evaluated once more by one of the original referees who finds that you have satisfactorily addressed the remaining concerns and it is now suitable for publication. I am therefore happy to accept your manuscript for publication in The EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

© European Molecular Biology Organization