THE EMBO JOURNAL

Manuscript EMBO-2011-76970

Symmetry-Free Cryo-EM Structures of the Chaperonin TRiC Along its ATPase-Driven Conformational Cycle

Yao Cong, Gunnar F. Schröder, Anne S. Meyer, Joanita Jakana, Boxue Ma, Matthew T. Dougherty, Michael F. Schmid, Stefanie Reissmann, Michael Levitt, Steven L. Ludtke, Judith Frydman and Wah Chiu

Corresponding author: Wah Chiu, Baylor College of Medicine

Review timeline:

Submission date:	11 January 2011
Editorial Decision:	01 March 2011
Revision received:	14 July 2011
Editorial Decision:	16 August 2011
Revision received:	09 September 2011
Accepted:	14 September 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 01 March 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Let me first of all apologise for the exceptionally long delay in getting back to you with a decision. Unfortunately, we experienced difficulties in finding suitable and willing referees for this manuscript. In addition, one of the referees was not able to return his/her report as quickly as initially expected.

In the meantime, three referees have evaluated the manuscript, and their comments are shown below. As you will see while all three referees consider the manuscript as interesting and important in principle, they all raise concerns regarding the conclusiveness of the data - in particular in respect to the eight fold symmetry that you applied. On balance, we should be able to give you the chance to address these concerns in a revised version of the manuscript. However, it will be indispensable to address the referees' concerns in a fully convincing manner and to their full satisfaction. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript as well as on the final assessment by the referees.

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance, and we may be able to grant an extension.

When preparing your letter of response to the referees' comments, please bear in mind that this will

form part of the Peer 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

The manuscript "A structural view of the conformational cycle of TRiC reveals an asymmetric intermediate induced by ATP hydrolysis" by Cong et al. reports 5 cryo EM structures of the eukaryotic chaperonin TRiC/CCT representing different nucleotide-bound states of the reaction cycle.

TRiC/CCT is an essential component of the eukaryotic folding machinery thought to be required for the folding of ~10 % of cellular proteins. It consists of two eight-membered rings stacked back to back. Each ring contains 8 distinct subunits, apparently arranged in a fixed topology. Like its bacterial homolog GroEL, TRiC/CCT is known to cycle through open and closed states in a nucleotide-dependent manner. Open and closed conformations differ by the relative orientations of the subunit domains: An equatorial domain harboring the nucleotide binding site, an intermediate domain and an apical domain close to the pore. In contrast to GroEL, TRiC/CCT has an inbuilt lid that consists of a helical segment protruding from the apical domains towards the pore at each apex. Closing of the pore is thought to be induced by the transition state of ATP hydrolysis. Negative cooperativity between the rings is thought to prevent simultaneous closure of both rings under physiological conditions.

Recently, the first high resolution structures of the TRiC/CCT complex were published. The first was a 4 Å EM structure of a fully closed complex in the presence of ATP•AlFx by the same authors, the second a 5.5 Å crystal structure of a tubulin substrate complex with the chaperonin in a fully open conformation (Munoz et al., NSMB).

The authors present now the structures of apo-TRiC and the complexes with ADP, AMP-PNP and ADP•AlFx at relatively high resolution (9-10 Å). Furthermore, an ATP•AlFx structure at higher resolution is shown, resembling the previously published structure. Importantly, the apo and ADP•AlFx structures are asymmetric, i.e. the two rings have distinct conformations. In apo- TRiC, one ring resembles the structures found for ADP and AMP-PNP, the other is somewhat more open with a larger pore. In the ADP•AlFx complex, one ring appears almost fully closed, the other is more open than the open ring of the apo-structure. Interestingly, the closed ring surrounds a cavity that is much larger than in the ATP•AlFx complex, and might be able to enclose a substrate protein up to 70 kDa. The authors explain the structural transitions by rotations of the TRiC domains, which ultimately result in different orientations of the helical protrusions and the distinct conformations in the two rings. How these rotations are induced by ATP hydrolysis and structurally transmitted between the domains and subunits remains unclear. Contact points between the subunits, which might stabilize the observed conformations are described.

These are interesting and novel findings, but the following points should be addressed prior to publication.

Specific points:
1. One important point that needs clarification concerns the use of eight-fold averaging, which was applied despite the known heteromeric ring structure and the grossly different subunit conformations observed in the crystal structure. This suggests that the apical domains have considerable degrees of

freedom in the open conformation, which might result in a distorted structure after averaging. A possible indication for this appears to be the electron density between the apical domains, which is unaccounted for by the atomic model (comparing the lower contact in Figure 5B left and middle). Does particle classification suggest a marked heterogeneity of apical domain orientations? Can a rotational self-correlation function be calculated that represents only the pore region? Here one could use the ATP•AlFx complex as a reference, which is the most compact conformation.

2. Are the observed contacts shown in Figure 5B sequence-conserved between TRiC subunits? These seem to bridge rather large distances in some conformations: What kind of molecular interactions might these be (salt bridges, van-der-Waals interactions, pi stacking)?

3. It is sometimes difficult to understand what was exactly overlayed / superposed, when comparisons are made: In Figures 3D and 6, no portion of the shown molecules seems to match well. Instead it appears that there is an overall rotation (Figure 3D) or downward shift (Figure 6). Was the complete hexadecameric complex superposed? Which program was used? Which portion of the molecule fitted best? Was there a distance cut-off used to exclude portions that do not fit?

4. In Figure 6, secondary structure elements should be indicated for orientation. In the main text, helices H4, H5, H12, H13 and H14 are mentioned in this context.

Referee #2

This paper describes C8-symmetrized cryo-EM structures of chaperonin TRiC/CCT in different nucleotide states at resolutions around 1nm (apart from the previously reported ATP-AlFx state at 4 Angstrom). Interestingly, the TRiC-ADP-AlFx state shows a mixture of two structures, of which the predominant structure is asymmetric: with one ring closed and one ring open. The authors used a flexible fitting approach to propose pseudo-atomic models for the different states. Based on these models, they propose models for the chamber closing mechanism and the negative inter-ring cooperativity of TRiC.

Compared to older cryo-EM work (see citations in the paper), the structures presented are of significantly higher resolution, and the collected data (25,000-35,000 particles per reconstruction) seem to be of much improved quality. Therefore, it strikes me that C8 symmetry was still applied in this study: the symmetrization was obviously a poor-man's solution for the refinement of low-resolution data in the older studies. TRiC has 8 different subunits, and last year the same authors already presented an asymmetric structure of the ATP-AlFx state at a similar resolution. Moreover, the application of C8 symmetry on each of the nucleotide states implies the assumption of a concerted mechanism for nucleotide binding: i.e. either all 8 or none of the subunits in each ring bind the nucleotide. A recent crystal structure of TRiC in complex with substrate at 5.5 Angstrom (Munoz et al 2010) showed very strong deviations from C8 symmetry, and anomalous difference maps of crystals grown in the presence of methylseleno-ATP-gamma-S indicated that only a few of the 16 subunits were bound to the nucleotide, indicating that nucleotide binding in TRiC does not happen in a concerted manner.

Therefore, I think it is unacceptable that C8 symmetry has been applied in this study. In general, the application of incorrect symmetry during cryo-EM refinements may lead to important artefacts in the reconstructed maps, although the refinement statistics (such as the Fourier-shell correlation) may actually improve upon the application of symmetry. Indications that these maps may indeed suffer from artefacts is the rather smooth appearance of the equatorial domains in the side views of Figure 2, especially in panels A, B and C. Also, Suppl. Figure 3 does not look convincing : there are important breaks in density for helices, and important connections between them. Given that the authors have proposed pseudo-atomic models for each of the states, it would be interesting to see FSC-curves as calculated between the maps and the atomic models: large difference between these curves and the ones calculated between random-halves of the cryo-EM data would be an indication of problems.

An asymmetric structure for the ATP-AlFx state was already presented at a similar resolution by the same group, so the symmetrised version of that structure in this paper is of no value whatsoever. Regarding the other states, reconstructions should be made without the application of any symmetry,

even if this would lead to much lower resolutions. Only after the asymmetric reconstructions have shown convincing indications for C8-symmetry, could that symmetry then be applied during the refinement. Without this test, the current structures cannot be relied upon, and the detailed analysis of the pseudo-atomic models is of little value.

Other comments:

In my opinion, the sentence "Although several high resolution structures of archaeal chaperonins are avalaible (refs), the high resolution structure of the TRiC complex, especially in the crucial substrate folding state, is still lacking to reveal its specificity" (p. 3), does injustice to the crystal structure by Munoz et al (2010).

It is completely unclear to me why one would build an asymmetric homology model (p.5) if this model is going to be docked in a symmetrized map.

Referee #3

Cong and colleagues have performed a comprehensive structural analysis of the conformational changes of the eukaryotic group II chaperonin TRiC/CCT throughout the ATPase cycle. Group II chaperonins are essential for proper folding of many proteins and defects in protein folding are associated with certain diseases. Several X-ray structures and cryo-EM structures of group II chaperonins are available. Nevertheless, the mechanism how ATP binding and ATPase are related to the conformational cycle of TriC to facilitate protein folding remains unclear. In order to close this gap, the authors present cryo-EM maps of bovine TRiC in five different nucleotide binding states, including the apo state, the ATP and ADP-bound states and the two ATPase transition states induced by ADP-AlFx and ATP-AlFx. The present study aims at closing this gap and is potentially very interesting. The authors show evidence for two conformational populations in the TRiC-ADP-AlFx complex and present an important 3D map of one conformer with one ring open and the other closed. However, it is not clear, if the resolution obtained is good enough to substantiate smaller differences between the maps. The authors emphasize the sub-nanometer resolution of their maps, but the nominal resolution is not much better than 10 A (9.2 -9.8; only TRiC-ATP-AlFx is at 4.2 A). A critical feature of sub-nanometer resolution is the appearance of α-helices as rod-like density. Unfortunately, α-helices in general appear not resolved. This is obvious in the side views of Fig. 2 and 3. Thus, it would be desirable to improve the maps to a real sub-nanometer resolution (around 8 A) with clearly resolved α-helices.

Specific points:

1. All the maps should be shown in solid surface representation without models.
2. Comparison of maps in figures and movies is done using fitted molecular models. As the experimental data are cryo-EM maps at intermediate resolution and not X-ray maps, it is crucial that the conformational changes are also demonstrated directly at the level of the cryo-EM densities.
3. It is not discussed why the resolution of TRiC-ATP-AlFx is much better than the resolution of the other states despite a similar number of particle images used. Maybe, sample heterogeneity has to be addressed in all samples.
4. Why the second cryo-EM map of ADP-AlFx sample after multirefine is not shown?

Minor Points:

5. In figure 2 the apical domain helical protrusions should be indicated.
6. Supplementary Movie 1 is quite long. It is unclear where the 8 A difference in the diameter of the cis ring is shown (TriC-ADP-AlFx versus TRiC-ATP-AlFx; page 8, 3rd paragraph).
7. How many micrographs have been used for the reconstructions?

1st Revision - authors' response 14 July 2011

Referee #1

The manuscript by Cong et al. reports 5 cryo EM structures of the eukaryotic chaperonin TRiC/CCT representing different nucleotide-bound states of the reaction cycle.

TRiC/CCT is an essential component of the eukaryotic folding machinery thought to be required for the folding of ~10 % of cellular proteins. It consists of two eight-membered rings stacked back to back. Each ring contains 8 distinct subunits, apparently arranged in a fixed topology. Like its bacterial homolog GroEL, TRiC/CCT is known to cycle through open and closed states in a nucleotide-dependent manner. Open and closed conformations differ by the relative orientations of the subunit domains: An equatorial domain harboring the nucleotide binding site, an intermediate domain and an apical domain close to the pore. In contrast to GroEL, TRiC/CCT has an inbuilt lid that consists of a helical segment protruding from the apical domains towards the pore at each apex. Closing of the pore is thought to be induced by the transition state of ATP hydrolysis. Negative cooperativity between the rings is thought to prevent simultaneous closure of both rings under physiological conditions.

Recently, the first high resolution structures of the TRiC/CCT complex were published. The first was a 4 Å EM structure of a fully closed complex in the presence of ATP-AlFx by the same authors, the second a 5.5 Å crystal structure of a tubulin substrate complex with the chaperonin in a fully open conformation (Munoz et al., NSMB).

The authors present now the structures of apo-TRiC and the complexes with ADP, AMP-PNP and ADP-AlFx at relatively high resolution (9-10 Å). Furthermore, an ATP-AlFx structure at higher resolution is shown, resembling the previously published structure. Importantly, the apo and ADP-AlFx structures are asymmetric, i.e. the two rings have distinct conformations. In apo- TRiC, one ring resembles the structures found for ADP and AMP-PNP, the other is somewhat more open with a larger pore. In the ADP-AlFx complex, one ring appears almost fully closed, the other is more open than the open ring of the apo-structure. Interestingly, the closed ring surrounds a cavity that is much larger than in the ATP-AlFx complex, and might be able to enclose a substrate protein up to 70 kDa. The authors explain the structural transitions by rotations of the TRiC domains, which ultimately result in different orientations of the helical protrusions and the distinct conformations in the two rings. How these rotations are induced by ATP hydrolysis and structurally transmitted between the domains and subunits remains unclear. Contact points between the subunits, which might stabilize the observed conformations are described.

These are interesting and novel findings, but the following points should be addressed prior to publication.

Specific points:
QI-1. One important point that needs clarification concerns the use of eight-fold averaging, which was applied despite the known heteromeric ring structure and the grossly different subunit conformations observed in the crystal structure. This suggests that the apical domains have considerable degrees of freedom in the open conformation, which might result in a distorted structure after averaging. A possible indication for this appears to be the electron density between the apical domains, which is unaccounted for by the atomic model (comparing the lower contact in Figure 5B left and middle).

AI-1. We agree fully with this reviewer that imposed symmetry in the reconstruction would not reveal the entire truth. We performed the reference-free 2-D image analysis, which revealed an asymmetric feature and the highly dynamic nature of TRiC especially in the open conformational states (Supplementary Movie 1). Therefore, we carried out symmetry-free reconstructions on all the states and made extensive revisions in the revised manuscript.

Our symmetry-free cryo-EM maps revealed an asymmetric pattern among the eight distinct subunits of TRiC especially in the open conformational states and a new intra- and inter-ring subunits interaction framework not seen before. However, the overall conclusion from our previous manuscript still holds, such as the existence of the one-ring closed intermediate of TRiC in the ATP hydrolysis transition state. These new structures allowed us to describe the mechanisms of TRiC inter-ring subunit negative cooperativity, intra-ring subunit positive cooperativity and chamber

closure in more detail. We have modified all the related figures (Figures 2-6 and related Supplementary Figures), movies and texts (highlighted in blue) in the revised manuscript.

QI-2. Does particle classification suggest a marked heterogeneity of apical domain orientations?

AI-2. Based on the added reference-free 2-D image analysis and the symmetry-free 3-D reconstructions of the open conformational states (Figures 2), the apical-domain orientation varies (Supplemental Figure 3B) and some of the subunits (e.g. a_1) are extremely dynamic especially in the apo state in solution (Supplementary Movie 1). This kind of continuous conformation variation in some regions of the complex is similar to the regions with high B-factor in X-ray crystallographic map, which makes high resolution structure determination more difficult. Interestingly, in the presence of nucleotide, such as in the AMP-PNP and ADP states, the extremely dynamic subunit a_1 is stabilized and better resolved (Supplementary Movie 1B-C, Figures 2B-C).

QI-3. Can a rotational self-correlation function be calculated that represents only the pore region? Here one could use the ATP-AlFx complex as a reference, which is the most compact conformation.

AI-3. Yes, we performed the rotational self-correlation calculation of the complete TRiC complex, instead of only the pore region, for the three open conformational states (Supplementary Figure 7). Since no symmetry was imposed in the reconstruction process, this correlation calculation clearly demonstrates how asymmetric TRiC is especially in the apo and AMP-PNP states, while in the ADP states TRiC appears to become more symmetrical. Thus there is no need to calculate the self-correlation function for only the pore region.

QI-4. Are the observed contacts shown in Figure 5B sequence-conserved between TRiC subunits?

AI-4. In our current symmetry-free maps, the intra-ring subunit contacts of TRiC are more complex than the previous symmetry-imposed maps. In the closed ring, the intra-ring apical domain contacts exist in most of the neighboring subunits (Figure 3A-B); in the open conformational states, such interactions mostly exist between pairs of neighboring subunits, generating unexpected pseudo-four-fold symmetry (e.g. Figure 5B-C). Therefore, our general conclusion about the intra-ring apical domain interaction holds but with more complicated features. We have modified the text in the last paragraph in P. 10. In addition, we checked the sequence alignment of all the eight TRiC subunits. There is no identical conserved residue/sequence but some semi-conserved substitutions in the intra-ring contacting regions. This indicates that the interaction may not be between exactly the same residues for the subunit pairs but may likely be between residues with similar chemical properties. In addition, the interaction strength may vary in different subunit pairs as seen in the open states (Figure 5B-C).

QI-5. These seem to bridge rather large distances in some conformations: What kind of molecular interactions might these be (salt bridges, van-der-Waals interactions, pi stacking)?

AI-5. In our new symmetry-free reconstructions, due to the dynamic nature of the apical domain, the preferred orientation problem and structural heterogeneity associate with some states of TRiC, most of our maps are at rather low resolution (10.5-13.9 Å). Therefore, we decide to omit the detailed interpretation on the intra-ring interactions in terms of fitted models as depicted in old Figure 5 in the previous manuscript. However, in the closed conformation, the interactions in this region are predominately salt-bridges. Of note, as discussed in AI-4, the apical domain interactions hold in majority of these paired subunits.

QI-6. It is sometimes difficult to understand what was exactly overlayed / superposed, when comparisons are made: In Figures 3D and 6, no portion of the shown molecules seems to match well. Instead it appears that there is an overall rotation (Figure 3D) or downward shift (Figure 6). Was the complete hexadecameric complex superposed? Which program was used? Which portion of the molecule fitted best? Was there a distance cut-off used to exclude portions that do not fit?

AI-6. When comparing two states, the maps of the entire TRiC complex of the two states were superimposed to each other using the "*Fit in Map*" module in CHIMERA; then the corresponding models are fit into the corresponding maps respectively using also the "*Fit in Map*" module in CHIMERA. This way the complete TRiC complex models were superimposed to each other.

Equatorial and intermediate domains fit the best between the map and the model. The apical domain fits less well due to its dynamic property. It is our experience in other projects that the fitting is less certain for highly dynamic region. In the present flexible fitting with the new symmetry-free maps, due to the density missing of some subunits especially in the apo state, the model in those portions was not included (Supplementary Figure 3).

QI-7. In Figure 6, secondary structure elements should be indicated for orientation. In the main text, helices H4, H5, H12, H13 and H14 are mentioned in this context.

AI-7. In the revised version, we eliminated the original Figure 6 because of the lower resolution maps now reported for the symmetry-free reconstructions. Therefore, there is no need to indicate the location of those SSEs.

Referee #2

This paper describes C8-symmetrized cryo-EM structures of chaperonin TRiC/CCT in different nucleotide states at resolutions around 1nm (apart from the previously reported ATP-AlFx state at 4 Angstrom). Interestingly, the TRiC-ADP-AlFx state shows a mixture of two structures, of which the predominant structure is asymmetric: with one ring closed and one ring open. The authors used a flexible fitting approach to propose pseudo-atomic models for the different states. Based on these models, they propose models for the chamber closing mechanism and the negative inter-ring cooperativity of TRiC.

QII-1. Compared to older cryo-EM work (see citations in the paper), the structures presented are of significantly higher resolution, and the collected data (25,000-35,000 particles per reconstruction) seem to be of much improved quality. Therefore, it strikes me that C8 symmetry was still applied in this study: the symmetrization was obviously a poor-man's solution for the refinement of low-resolution data in the older studies. TRiC has 8 different subunits, and last year the same authors already presented an asymmetric structure of the ATP-AlFx state at a similar resolution.

AII-1. As discussed in AI-1 above, we followed the suggestion from both the reviewers 1 and 2 to provide symmetry-free reconstructions for all the apo and nucleotide-bound states. We did collect more data than the other previous cryo-EM studies on TRiC, which allowed us to obtain reconstructions without imposing any symmetry.

In our current manuscript version, the primary reasons of the lower resolutions achieved in the four states (10.5-13.9 Å) relative to the both-ring closed TRiC-ATP-AlFx at 4.7 Å resolution are likely attributable to 1) the extremely dynamic character of TRiC (Supplementary Movie 1), 2) the particle preferred orientation problem in these four states, and 3) the structure heterogeneity (in ADP-AlFx state, Supplementary Figure 1A-B). These phenomena are well known in the cryo-EM studies on group II chaperonins (Clare et al, 2008; Zhang et al, 2010). As for TRiC-ATP-AlFx, it does not suffer from any of the above problems, and we are able to obtain a map at 4.7 Å resolution. We have expanded the discussion on the resolution difference among these structures in our manuscript (paragraph one on P. 5).

QII-2. Moreover, the application of C8 symmetry on each of the nucleotide states implies the assumption of a concerted mechanism for nucleotide binding: i.e. either all 8 or none of the subunits in each ring bind the nucleotide. A recent crystal structure of TRiC in complex with substrate at 5.5 Angstrom (Munoz et al 2010) showed very strong deviations from C8 symmetry, and anomalous difference maps of crystals grown in the presence of methylseleno-ATP-gamma-S indicated that only a few of the 16 subunits were bound to the nucleotide, indicating that nucleotide binding in TRiC does not happen in a concerted manner.

AII-2. In the revised manuscript, we released the symmetry in our reconstructions and revealed the asymmetric feature of TRiC in all the five biochemical states. This indicates that the nucleotide binding/hydrolysis ability might be different in different TRiC subunits. This is an interesting and important question. In the revised manuscript, we have focused on the overall TRiC structural variations in different biochemical states. Due to the resolution limitation of our current maps, we are reluctant to discuss the nucleotide binding modes.

QII-3. Therefore, I think it is unacceptable that C8 symmetry has been applied in this study. In general, the application of incorrect symmetry during cryo-EM refinements may lead to important artefacts in the reconstructed maps, although the refinement statistics (such as the Fourier-shell correlation) may actually improve upon the application of symmetry.

AII-3. We followed the suggestion from the reviewers to provide new symmetry-free reconstructions of TRiC in all the five biochemical states in the revised manuscript (Fig. 2-3), please also refer to AI-1.

QII-4. Indications that these maps may indeed suffer from artefacts is the rather smooth appearance of the equatorial domains in the side views of Figure 2, especially in panels A, B and C. Also, Suppl. Figure 3 does not look convincing: there are important breaks in density for helices, and important connections between them.

AII-4. In the current manuscript version, all the maps without imposed symmetry are done in the 10.5-13.9 Å resolution range without seeing the helices and sheets. Therefore, we removed the old Figure 2 and Supplementary Figure 3.

QII-5. Given that the authors have proposed pseudo-atomic models for each of the states, it would be interesting to see FSC-curves as calculated between the maps and the atomic models: large difference between these curves and the ones calculated between random-halves of the cryo-EM data would be an indication of problems.

AII-5. We report the conventional FSC curves for all the four states by splitting the datasets into two halves as shown in Supplementary Figure 2B.

QII-6. An asymmetric structure for the ATP-AlFx state was already presented at a similar resolution by the same group, so the symmetrised version of that structure in this paper is of no value whatsoever.

AII-6. In the revised manuscript, we included the asymmetric structure of the ATP-AlFx state (Cong et al, 2010) only to facilitate the visualization of the TRiC conformational transitions throughout the ATP cycle in a more complete manner. We clarify this point in the text.

QII-7. Regarding the other states, reconstructions should be made without the application of any symmetry, even if this would lead to much lower resolutions. Only after the asymmetric reconstructions have shown convincing indications for C8-symmetry, could that symmetry then be applied during the refinement. Without this test, the current structures cannot be relied upon, and the detailed analysis of the pseudo-atomic models is of little value.

AII-7. As discussed in AI-1 and AII-1, in the revised manuscript, we have provided the symmetry-free reconstructions of TRiC in the five states throughout the ATPase cycle.

Other comments:

QII-8. In my opinion, the sentence "Although several high resolution structures of archaeal chaperonins are available (refs), the high resolution structure of the TRiC complex, especially in the crucial substrate folding state, is still lacking to reveal its specificity" (p. 3), does injustice to the crystal structure by Munoz et al (2010).

AII-8. We have modified this statement (second paragraph on P. 3).

QII-9. It is completely unclear to me why one would build an asymmetric homology model (p.5) if this model is going to be docked in a symmetrized map.

AII-9. We now report symmetry-free maps at low resolution. We have compared the crystal structure model (Munoz et al, 2011) with our map to validate our map (Supplementary figure 5). In the supplementary figure 3, we presented our models for all the five states to convey the structural complexity more readily and make them easier to comprehend. Note that we have bundled the map

and model together in the illustrative figures and movies as suggested by reviewer 3 (see AIII-4). We eliminate detailed discussions solely based on the models as done in the previous paper because the low resolution fitted models are inherently imprecise.

Referee #3

Cong and colleagues have performed a comprehensive structural analysis of the conformational changes of the eukaryotic group II chaperonin TRiC/CCT throughout the ATPase cycle. Group II chaperonins are essential for proper folding of many proteins and defects in protein folding are associated with certain diseases. Several X-ray structures and cryo-EM structures of group II chaperonins are available. Nevertheless, the mechanism how ATP binding and ATPase are related to the conformational cycle of TriC to facilitate protein folding remains unclear. In order to close this gap, the authors present cryo-EM maps of bovine TRiC in five different nucleotide binding states, including the apo state, the ATP and ADP-bound states and the two ATPase transition states induced by ADP-AlFx and ATP-AlFx. The present study aims at closing this gap and is potentially very interesting.

QIII-1. The authors show evidence for two conformational populations in the TRiC-ADP-AlFx complex and present an important 3D map of one conformer with one ring open and the other closed. However, it is not clear, if the resolution obtained is good enough to substantiate smaller differences between the maps.

AIII-1. First of all, difference between the both-ring open and only one-ring closed conformations of TRiC has been distinguished in the reference-free 2-D image analysis of the raw particles of the TRiC-ADP-AlFx dataset (Supplementary Figure 1A-B). This clearly demonstrates that the EMAN image processing software utilized here is powerful enough to capture those conformation variations within this dataset. In addition, our reported maps were carried out at 13.9 Å for the one-ring closed conformation and ~16 Å for the both ring open conformation in this dataset, which is sufficient to capture the conformational difference between the two maps. For instance, when comparing the open ring with the closed ring, the central hole diameters differ more than 20 Å. Therefore, the conformational difference between the two conformational populations in the TRiC-ADP-AlFx dataset is larger than the claimed resolution such that our observation is believable and we do not over-interpret our maps.

QIII-2. The authors emphasize the sub-nanometer resolution of their maps, but the nominal resolution is not much better than 10 A (9.2 -9.8; only TRiC-ATP-AlFx is at 4.2 A). A critical feature of sub-nanometer resolution is the appearance of a-helices as rod-like density. Unfortunately, a-helices in general appear not resolved. This is obvious in the side views of Fig. 2 and 3. Thus, it would be desirable to improve the maps to a real sub-nanometer resolution (around 8 A) with clearly resolved a-helices.

AIII-2. In the revised version, we have replaced all the symmetrized maps with the symmetry-free reconstructions (Figures 2-3) determined in the 10.5-13.9 Å resolution range. Therefore, we do not discuss subnanometer resolution structural details.

Specific points:

QIII-3. All the maps should be shown in solid surface representation without models.

AIII-3. We followed the suggestion from this reviewer. In the figures of revised version, we rendered all the maps in the surface representation without models (Figures 2-6).

QIII-4. Comparison of maps in figures and movies is done using fitted molecular models. As the experimental data are cryo-EM maps at intermediate resolution and not X-ray maps, it is crucial that the conformational changes are also demonstrated directly at the level of the cryo-EM densities.

AIII-4. We thank this reviewer for the suggestion. In the revised version, we generated the movies directly showing the morphing based on the density maps of different states (Supplementary Movie 2-4).

QIII-5. It is not discussed why the resolution of TRiC-ATP-AlFx is much better than the resolution of the other states despite a similar number of particle images used. Maybe, sample heterogeneity has to be addressed in all samples.

AIII-5. We have expanded the discussion on the resolution difference among these structures (paragraph one on P. 5; see AII-1).

QIII-6. Why the second cryo-EM map of ADP-AlFx sample after multirefine is not shown?

AIII-6. We thank this reviewer for the suggestion. In the revised manuscript, we have included the open state map, derived from ~35% of the particle population, from the ADP-AlFx dataset (Supplementary Figure 1C).

Minor Points:

QIII-7. In figure 2 the apical domain helical protrusions should be indicated.

AIII-7. We thank the reviewer for the suggestion. We've put some dotted lines to indicating the apical protrusion location and orientation (Supplemental Figure 3B).

QIII-8. Supplementary Movie 1 is quite long. It is unclear where the 8 A difference in the diameter of the cis ring is shown (TriC-ADP-AlFx versus TRiC-ATP-AlFx; page 8, 3rd paragraph).

AIII-8. Supplementary Movie 1 in the previous manuscript has been removed, and each of the transition between neighboring states has been replaced by Supplementary Movies 2-4, where all the nucleotide states have been indicated. Also, the chamber radius difference in the cis-ring is shown directly in figure 3C.

QIII-9. How many micrographs have been used for the reconstructions?

AIII-9. The raw particle number included in the final reconstruction for each state has been listed in Supplementary Figure 2A, which is about 75-80% of the original particle numbers.

References:

Clare DK, Stagg S, Quispe J, Farr GW, Horwich AL, Saibil HR (2008) Multiple states of a nucleotide-bound group 2 chaperonin. *Structure* **16:** 528-534

Cong Y, Baker ML, Jakana J, Woolford D, Miller EJ, Reissmann S, Kumar RN, Redding-Johanson AM, Batth TS, Mukhopadhyay A, Ludtke SJ, Frydman J, Chiu W (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

Munoz IG, Yebenes H, Zhou M, Mesa P, Serna M, Park AY, Bragado-Nilsson E, Beloso A, de Carcer G, Malumbres M, Robinson CV, Valpuesta JM, Montoya G (2011) Crystal structure of the open conformation of the mammalian chaperonin CCT in complex with tubulin. *Nat Struct Mol Biol* **18:** 14-19

Zhang J, Baker ML, Schroder GF, Douglas NR, Reissmann S, Jakana J, Dougherty M, Fu CJ, Levitt M, Ludtke SJ, Frydman J, Chiu W (2010) Mechanism of folding chamber closure in a group II chaperonin. *Nature* **463:** 379-383

2nd Editorial Decision 16 August 2011

Thank you for sending us your revised manuscript. Our original referees have now seen it again, and you will be pleased to learn that in their view you have addressed their criticisms in a satisfactory manner, and that the paper will therefore be publishable in The EMBO Journal.

Before this will happen, however, I was wondering whether you would like to consider addressing the minor issues mentioned by referee 2 (see below). Furthermore, could you please add an author contribution section to the main body of the manuscript text below the acknowledgements section and include the full accession details for the EMDB and PDB entries.

I will then formally accept the manuscript.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The revised manuscript "Symmetry-Free Cryo-EM Structures of Chaperonin TRiC Along its ATPase Driven Conformational Cycle" by Cong et al. presents a comprehensive view of the conformational cycle of the eukaryotic chaperonin TRiC/CCT. This includes the ADP-AlFx state, which has only one ring closed, a state postulated for TRiC/CCT based on the observed allostery of ATP hydrolysis.
This is an important work, complementary to the recent crystal structures of TRiC/CCT in the open state (Munoz et al., 2010) and the fully closed state (Dekker et al., 2011), showing the particle without the constraints of crystal packing.
The decision to omit 8-fold symmetry averaging in the revised version of the manuscript greatly paid off: Because no symmetry constraints were used, the individual subunits can now be discerned (although not assigned to specific sequences due to the limited resolution). The "unwrapped view" in Figure 4 gives an impression, how the contacts between subunits intensify along the rings on the trajectory towards ATP hydrolysis. A preferred pairwise interaction of adjacent subunits is described for the first time.
The main conclusions from the previous version of the manuscript on domain movements during the conformational cycle remain valid.
I strongly encourage publication of the revised version of the manuscript in EMBO Journal.

Referee #2 (Remarks to the Author):

The authors have recalculated their structures without imposing 8-fold symmetry. The pronounced asymmetry of the resulting structures confirms that symmetry averaging was not adequate in this study. However, due to the decreased number of asymmetric units in each reconstruction, the resolutions have now dropped to levels where the structures may only be interpreted as elongated blobs that form open or closed folding chambers with varying degrees of asymmetry. This is technically the only correct way of handling these data, but it is up to the editor to decide whether such structures are of sufficiently broad interest for publication in EMBO journal.

Minor points:

P6.
"Figure 4A shows that the line between subunit a3 in one ring and subunit a'7 in the other ring has a larger tilt angle and is less parallel (Figure 4A) to the lines joining other subunit pairs. We thus postulate that the dynamic subunit (a1) or the outward-tilting one (a3) might be critical to recruit substrate in solution. "
=> I do not see how this conclusion can be drawn from Figure 4A.

P7.

"Of note, the TRiC-AMP-PNP map demonstrates that subunit a1 is better resolved than its counterpart a'1 in the other ring (Figure 4B), suggesting that it is more suited for the lid closure in that ring because it requires all the subunits in one ring to be more symmetrically arranged. This may reflect the negative inter-ring cooperativity observed for ATP hydrolysis (Kafri et al, 2001; Reissmann et al, 2007) and may be linked to the "two-stroke" mechanism as previously proposed for TriC. "
=> I do not see how the observation of better defined density in one ring supports these conclusions.

P8.
" Using the correlation between this asymmetric TRiC-ADP-AlFx map and the TRiC-AMP-PNP map (Figure 5E), we can register their subunits in both rings (Figures 5G-H). This registration is validated by the match of the characteristic outward tilting subunits a3 and a'3 in the two maps (Figure 5G-H). "
=> This is a circular argument: the subunits a3 and a3' are part of the correlation calculation and cannot therefore "validate" it.

Referee #3 (Remarks to the Author):

Cong and colleagues have thoroughly revised the manuscript to address the criticism and concerns of the reviewer. The calculation of 3D maps without imposing symmetry apparently was a crucial step to improve the analysis. The nominal resolutions of the maps are now lower than the ones reported in the original manuscript but this does not matter, because the discussion of subnanometer structural details has been removed. The analysis of the differences of the maps and the underlying conformational changes of TRiC is therefore much more solid now.

2nd Revision - authors' response 09 September 2011

Referee #1

QI-1: The revised manuscript "Symmetry-Free Cryo-EM Structures of Chaperonin TRiC Along its ATPase Driven Conformational Cycle" by Cong et al. presents a comprehensive view of the conformational cycle of the eukaryotic chaperonin TRiC/CCT. This includes the ADP-AlFx state, which has only one ring closed, a state postulated for TRiC/CCT based on the observed allostery of ATP hydrolysis. This is an important work, complementary to the recent crystal structures of TRiC/CCT in the open state (Munoz et al., 2010) and the fully closed state (Dekker et al., 2011), showing the particle without the constraints of crystal packing. The decision to omit 8-fold symmetry averaging in the revised version of the manuscript greatly paid off: Because no symmetry constraints were used, the individual subunits can now be discerned (although not assigned to specific sequences due to the limited resolution). The "unwrapped view" in Figure 4 gives an impression, how the contacts between subunits intensify along the rings on the trajectory towards ATP hydrolysis. A preferred pairwise interaction of adjacent subunits is described for the first time. The main conclusions from the previous version of the manuscript on domain movements during the conformational cycle remain valid. I strongly encourage publication of the revised version of the manuscript in EMBO Journal.

AI-1: Thanks for the suggestions and complements from this Reviewer. We tried our best to carry out a more complete and thorough study on this complex chaperonin system, and hopefully our work can bring some useful information to the field.

Referee #2

The authors have recalculated their structures without imposing 8-fold symmetry. The pronounced asymmetry of the resulting structures confirms that symmetry averaging was not adequate in this study. However, due to the decreased number of asymmetric units in each reconstruction, the resolutions have now dropped to levels where the structures may only be interpreted as elongated blobs that form open or closed folding chambers with varying degrees of asymmetry. This is

technically the only correct way of handling these data, but it is up to the editor to decide whether such structures are of sufficiently broad interest for publication in EMBO journal.

Minor points:
QII-1: P6. "Figure 4A shows that the line between subunit a3 in one ring and subunit a'7 in the other ring has a larger tilt angle and is less parallel (Figure 4A) to the lines joining other subunit pairs. We thus postulate that the dynamic subunit (a1) or the outward-tilting one (a3) might be critical to recruit substrate in solution. " => I do not see how this conclusion can be drawn from Figure 4A.

AII-1: Thanks for the comment from the reviewer. In the revised version, we modified the figure (Figure 4A) and the description to make it more clear and specific (second paragraph on P. 6).

QII-2: P7. "Of note, the TRiC-AMP-PNP map demonstrates that subunit a1 is better resolved than its counterpart a'1 in the other ring (Figure 4B), suggesting that it is more suited for the lid closure in that ring because it requires all the subunits in one ring to be more symmetrically arranged. This may reflect the negative inter-ring cooperativity observed for ATP hydrolysis (Kafri et al, 2001; Reissmann et al, 2007) and may be linked to the "two-stroke" mechanism as previously proposed for TriC. " => I do not see how the observation of better defined density in one ring supports these conclusions.

AII-2: We have modified this statement (first paragraph on P. 7).

QII-3: P8. " Using the correlation between this asymmetric TRiC-ADP-AlFx map and the TRiC-AMP-PNP map (Figure 5E), we can register their subunits in both rings (Figures 5G-H). This registration is validated by the match of the characteristic outward tilting subunits a3 and a'3 in the two maps (Figure 5G-H). " => This is a circular argument: the subunits a3 and a3' are part of the correlation calculation and cannot therefore "validate" it.

AII-3: Thanks for the comment. In the revised version, we have modified this statement (second paragraph on P. 8).

Referee #3

QIII-1: Cong and colleagues have thoroughly revised the manuscript to address the criticism and concerns of the reviewer. The calculation of 3D maps without imposing symmetry apparently was a crucial step to improve the analysis. The nominal resolutions of the maps are now lower than the ones reported in the original manuscript but this does not matter, because the discussion of subnanometer structural details has been removed. The analysis of the differences of the maps and the underlying conformational changes of TRiC is therefore much more solid now.

AIII-1: Thanks for the suggestions and comments from Reviewer III, which led us to explore more thoroughly on the close to native conformational changes of TRiC.

