THE EMBO JOURNAL

Manuscript EMBO-2010-73926

Structural Basis for the Assembly and Gate Closure Mechanisms of the Mycobacterium Tuberculosis 20S Proteasome

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Review timeline:

Submission date:	11 February 2010
Editorial Decision:	05 March 2010
Revision received:	19 April 2010
Accepted:	20 April 2010

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 05 March 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received the comments of three reviewers, and I am pleased to inform you that all of them find your study interesting and in principle suitable for publication in The EMBO Journal. They nevertheless raise a number of specific issues that would need to be satisfactorily addressed prior to publication, including also some technical points raised by referee 2. In addition, several criticisms of all reviewers pertain to aspects of presentation and interpretation, including the sentiment that some of the conclusions on 'uniqueness' of the mycobacterial proteasome should be toned down.

I would therefore like to invite you to prepare a revised version of the manuscript, taking into account the various points raised by the referees (in this respect, please note that it will be important to diligently answer to all the points raised at this stage, as it is EMBO Journal policy to allow only a single round of major revision). Please also remember to include the PDB accession codes for the reported structures in this last version of the manuscript. Finally, when preparing your letter of response to the referees' comments, please also 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. In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Summary

This study shows xray and EM analysis of various intermediates of the assembly of the Mycobacterium tuberculosis (Mtb) 20S proteasome. Recombinant wild type and mutant Mtb proteasomes were purified from E. coli and allowed to mature under various conditions to capture different stages of proteasome assembly. The authors assert that the Mtb 20S core is unusual in that is assembled and gated differently from proteasomes of other organisms.

The study and structures are striking and reveal interesting new aspects for understanding proteasome assembly. However I have several comments that the authors should address or clarify before publication:

Major points:
1. p. 3, first paragraph: the authors state, "bacterial proteasomes are usually nonessential, because a number of other bacterial protease complexes, ..., perform similar functions". This is an assumption, and also not true since it is not known if all bacterial proteasomes are non-essential. In fact, the Mtb proteasome 20S is required for normal growth in vitro.

2. p. 3, last sentence: "High temperature growth (37 C)" is an incorrect statement and needs to be removed. 37 C is physiologic for both E. coli and Mycobacteria. Several statements throughout the manuscript suggest that proteasome assembly is influenced by various temperatures, and might represent physiologic conditions; however, Mtb never grows at room temperature because it is never found outside of a human host. Thus the suggestion the authors' in vitro temperature-dependent data might represent something relevant that happens in nature should be tempered.

3. p. 5, second sentence: I didn't understand what this meant, please clarify.

4. p. 13: last paragraph: "The long-standing conundrum of how to seal a converging space with 7 identical peptides..." I'm not sure this has been a long-standing "conundrum"; this statement should be toned down.

5. Section 5, p. 14: The crystal structure analysis using inhibitors is intriguing, but the authors never explain how the inhibitors work, where they bind, etc. This should be described even if published elsewhere. Also, it was not clear why the authors didn't use the wild type (closed) 20S proteasomes with the inhibitors (maybe this was explained in a previous paper, but should nonetheless be clarified). The analysis suggests that the H0 helix of the alpha subunit is flexible, but is this physiologic when 8 amino acids are deleted from the protein? The results of these experiments were also in the Discussion, and seemed a bit too speculative considering the artificiality of the system tested (i.e. OG proteasomes).

6. As I understand it, the authors suggest that the Mtb proteasome B-subunit propeptides "stick out", rather than into the catalytic core, but eventually these push their way into the core for processing. The authors further suggest that this is unique to Mtb and does not occur in the other bacterial proteasomes; however, has there been a similarly thorough analysis of proteasomes from related bacteria to suggest this process does not happen in these species? Please clarify. Along those lines p 15, Discussion - the authors state that the Mtb B-propeptide is a barrier to formation of full proteasome - why? Do the authors have any insight, are other factors involved, is this regulatory? Do the authors feel this happens in intact Mtb?

7. p 10, section 3 - please state the purpose of using T1A mutation and how it would inhibit maturation of proteasomes; although obvious to some readers, it might not be to all.

8. It should be more clearly defined as to what the "anterior chamber" is relative to a particular propeptide; also it is not clear where a peptide "ascends" as described here.

9. The observation that the alpha-subunits form an exceptionally tight gate is significant, and suggests that a unique mechanism may be required to activate/open it for polypeptide delivery. It was mentioned that Mpa does not robustly or stably interact with the Mtb 20S, suggesting it does not behave like a canonical eukaryotic or Archaeal proteasomal ATPase. Perhaps there should be some brief discussion about the presence of a HbYX motif in Mpa that would presumably be necessary and sufficient to activate the Mt 20S, yet does not.

10. Discussion: page 16: the second paragraph must be omitted. Mtb never grows outside of a mammalian host in nature (the authors implicated an environmental stage of Mtb-this never happens), thus one should presume that the proteasomes are synthesized and assembled at 37 degrees. To compare Listeria to Mtb is not appropriate, as Listeria is a true environmental organism that can also grow at a wide range of temperatures, whereas Mtb cannot.

11. P. 16, last paragraph: The second sentence seems a bit strong, I do not believe that other prokaryotic proteasomes were presumed to be ungated, just maybe less tightly (I might be wrong). I suggest that the authors simply state that the other proteasomes are not as tightly gated as the Mtb proteasome.

12. Last sentence of the discussion: I didn't understand what this meant. Please clarify and rephrase.

Minor points:
13. Rhodococcus should always be in italics.
14. p. 10: theB-propeptide needs to be separated.
15. The sections should not be numbered.
16. I would omit the word "Unique" from the title.

Referee #2 (Remarks to the Author):

The authors reported a number of structures, both by single particle cryoEM and X-ray crystallography, of mycobacterium tuberculosis (Mtb) 20S proteasome. These structures include a half proteasome, two different intermediates and fully assembled proteasome. There is a very large different in conformation of the beta-subunit between the half proteasome and the known atomic structure of Mtb 20S proteasome, indicating a large conformational change of the beta-subunit during the assembly process from half to full proteasome. Surprisingly, a structure corresponding to an intermediate state, where two half proteasome is shifted from each other by about 10A, was found. Together, these structures revealed the assembly process of Mtb 20S proteasome. Further, it revealed a closed gate of Mtb 20S proteasome in atomic details. Together, this manuscript presented a number of very interesting and exciting findings that will significantly enhance our understanding of assembly and gate closure mechanism of the Mtb 20S proteasome.

Specific comments:

In Figure 1, the authors assigned the density below the beta-ring to the beta propeptide. Although all the arguments supporting this assignment are very likely to be true, there is no direct evidence. Further, in the raw images, the propeptide seems very flexible. Considering that there is a large conformational change in the beta subunit from half proteasome to the IM-I and IM-II states, and the structure of the half proteasome is still relative low, there is a possibility that these densities are contributed by other factors, such as some part of the beta-subunit that was exposed at this conformation. Since having propeptide pointing outwards is one of the important findings of this manuscript, it is necessary to verify this assignment by, for example, using antibody or Fab that against the propeptide or using N-terminal conjugation with a GFP, or any other suitable methods.

Related to the IM-I state of Mtb proteasome, Figure 2A showed a number of class averages

representing different intermediate states between half (A1) and matured (A3) proteasome. It seems that there is a range of different intermediate states. But it is also possible that these slightly different class averages are different views of the same intermediate state. The 3D reconstruction of Fig.2 B and C was calculated by arresting particles in IM-I state homogeneously with inhibitor. Although at low resolution the 3D reconstruction looks similar with the class averages shown in Fig. 2A, it is not clear if the 3D reconstruction of Figure 2B truly represents an averaged intermediate state, or is deformed by inhibitor? Further, if the structure shown in Fig. 2C is just one of a serious intermediate states shown in Fig 2A2? The authors should address this issue.

The structure of IM-I is bit surprising. It is hard to think how in this conformation, where the beta-subunit of two half proteasomes are aligned and facing each other, could avoid steric clash of the protruding beta-propeptide. It is necessary to prove that this IM-I is indeed an intermediate state. One possible approach is to examine by negative stain EM the ratio of particles between IM-I and matured proteasome during the assembly process. One should find more IM-I particles in the beginning and fewer in the late of the process.

The side view of class average of T1A mutant half proteasome (Supplement Fig3C) looks quite different from the pai shaped low temperature cultured half proteasome (Fig1A). And it is hard to see if the propeptide is out of the half proteasome as shown in the Figure 1A. If it goes through the same assembly process, intermediate states should also be seen. Can author comment on this? How does T1A mutant look like, if the authors culture it at low temperature?

Minor points:

Applying a 2-fold symmetry in determining the 3D cryo-EM map of the Mtb 20S IM-I is still questionable. The side views of class averages do not have obvious 2-fold symmetry. In the 3D reconstruction, the half-proteasome part also lost its original 7-fold symmetry (Fig. 2C1). The authors should test if a 3D reconstruction without 2-fold symmetry indeed showing 2-fold symmetry, before applying it.

In Fig. 1A, the densities pointed by red arrows are very weak, almost at the level of noise background. Maybe variance of this region will show clearer density of the propeptide?

Suggest removing the word "unique" from the title.

Page 12, correct 20S IM-I to 20S IM-II
Page 15, correct Fig.5E and F to Fig.5D and E.
Page 20, Figure legend G and F to G and H

Referee #3 (Remarks to the Author):

The report by Li and colleagues reports a number of novel EM and X-ray structures for the Mycobacterium tuberculosis 20S proteasome and its assembly intermediates. These structures reveal 1) unusual positioning and changes in position of the 56-residue beta propeptide that seem to correlate with effects on assembly; 2) interesting dynamic changes in subunit conformation and relative positioning between the mature 20S proteasome and its precursors; and 3) novel structural features of the alpha-ring gate that allows a tight seal of the pore, at least in the precursor-like form called Mtb 20S IM-II.

Overall, I feel these structures provide substantial insight into the structural diversity of 20S proteasomes and the states they can go through in the process of assembly. The novel features of this work make it suitable for publication in the EMBO Journal. The paper is generally well written, with clear figures, and the data appear to be solid, although I am not a structural biologist. The one complaint I have is the unnecessary attempt to cast the Mtb 20S as extremely different from all previously studied 20S proteasomes. I think earlier work on the Rhodococcus and Thermoplasma proteasomes suggests otherwise. I would like to see these similarities represented more accurately in the final version of the paper, as suggested below.

The authors continually suggest that the Mtb beta propeptide has a uniquely inhibitory effect on

proteasome assembly. I think the similarities to the other well studied actinomycete proteasome, that from Rhodococcus, may be greater than the differences. First, it should be noted that in the Zuhl et al. (1997) reference, it was found that the Rhodococcus beta propeptide supplied in trans allowed far more rapid dimerization of half proteasomes compared to normal beta subunits with the propeptide in cis, consistent with the idea that the propeptide has to get repositioned or processed for stable dimerization to occur. This is reminiscent of the negative effect on dimerization suggested here. This likely similarity of the two actinomycete proteasomes should be noted. Second, the propeptide structure in preholoproteasome or 20S IM-II intermediate here is, as the authors point out, similar to the structure seen with the analogous intermediate of the Rhodococcus proteasome. Although the idea that the temperature block to assembly of the Mtb proteasome is related to the function of the proteasome in the M. tuberculosis life cycle, the comparable temperature shift has not been done with Rhodococcus, to my knowledge.

Similarly, the authors state that archaeal proteasomes have a partially open gate or are not gated, but I think this view is dated. The Rabl et al. (2008) paper, for example, shows clear electron density in the pore region of the T. acidophilum 20S proteasome, and there is definitely gating behavior of this proteasome through PAN binding. The slightly open gate might have more to do with how the proteasomes are isolated and purified than real differences in gating properties. At most, the difference is one of degree.

The experimental details are a little thin. For example, culture volumes or grams of wet cell pellets that were harvested should be given, as should protein concentrations, buffer volumes and column sizes. In general, the reader should be able to know how to replicate exactly what was done in the paper without having to contact the authors.

1st Revision - authors' response 19 April 2010

Responses to reviewers' comments

Referee #1

We thank the reviewer for the numerous suggestions that have significantly improved our manuscript.

Major points:
1. p. 3, first paragraph: the authors state, "bacterial proteasomes are usually nonessential, because a number of other bacterial protease complexes, ..., perform similar functions". This is an assumption, and also not true since it is not known if all bacterial proteasomes are non-essential. In fact, the Mtb proteasome 20S is required for normal growth in vitro.

The original sentence has been changed to: "In bacteria, a number of other protease complexes, such as HslUV and ClpAP, perform functions similar to that of the proteasomes".

2. p. 3, last sentence: "High temperature growth (37 ∞C)" is an incorrect statement and needs to be removed. 37 ∞C is physiologic for both E. coli and Mycobacteria. Several statements throughout the manuscript suggest that proteasome assembly is influenced by various temperatures, and might represent physiologic conditions; however, Mtb never grows at room temperature because it is never found outside of a human host. Thus the suggestion the authors' in vitro temperature-dependent data might represent something relevant that happens in nature should be tempered.

"High temperature growth (37 ∞C)" has been changed to "Growth at 37 ∞C" in revision throughout the manuscript. The original third paragraph in Discussion section speculating on potential physiological significance also has been deleted in revision.

3. p. 5, second sentence: I didn't understand what this meant, please clarify.

Filling a converging space such as the proteasome gate at the seven-fold symmetry axis requires a

symmetry breakdown, i.e. the N-termini of the seven identical alpha subunits have to take on different structures, in order to avoid overlap at the symmetry axis [See, for example, Jane Richardson, The Anatomy and Taxonomy of Protein Structure]. This situation is similar to protein packing in a spherical icosahedral virus in which subunit "quasi-equivalence" is required at the vortex (symmetry axes) [see D. Caspar, Movement and Self Control in Protein Assemblies ñ Quasi-Equivalence Revisited, Biophysical J, 1980].

Since the structural solution of the closed gate of the yeast proteasome with seven different alpha-subunit, thus naturally different N-terminal peptides that enabled sealing of the gate at the pseudo seven symmetry axis (Groll, et al., Nat Struct Bio, 2000), the question posed then and standing till now has been how and what kind of different structures the seven identical N-terminal peptides in the prokaryotic proteasomes would assume if the gate were closed. Our new structure answers question.

To clarify, we have deleted "long-standing" in revision, and have added "at the seven-fold symmetry axis". The revised sentence is: "By visualizing the N-terminal -octapeptide in the Mtb 20S proteasome structure, we elucidate how a homomeric proteasome is able to tightly seal its substrate entrance at the seven-fold symmetry axis with seven identical -octapeptides."

4. p. 13: last paragraph: "The long-standing conundrum of how to seal a converging space with 7 identical peptides..." I'm not sure this has been a long-standing "conundrum"; this statement should be toned down.

See clarification in our response to the reviewer's comment #3. In revision, "The long standing conundrum" is replaced by "The question of"

5. Section 5, p. 14: The crystal structure analysis using inhibitors is intriguing, but the authors never explain how the inhibitors work, where they bind, etc. This should be described even if published elsewhere. Also, it was not clear why the authors didn't use the wild type (closed) 20S proteasomes with the inhibitors (maybe this was explained in a previous paper, but should nonetheless be clarified). The analysis suggests that the H0 helix of the alpha subunit is flexible, but is this physiologic when 8 amino acids are deleted from the protein? The results of these experiments were also in the Discussion, and seemed a bit too speculative considering the artificiality of the system tested (i.e. OG proteasomes).

One sentence is inserted to explain the inhibitor: "The inhibitor GL1 was shown previously to covalently modify The-1, the active site residue of Mtb 20S, and introduce structural changes around the substrate binding pocket in the -subunit, and such mechanism of inhibition was not observed in the human proteasome (Lin et al, 2009)".

As to the question of why the wild type (closed) 20S was not used and we instead used the 20SOG mutant, this was so because our question was not if inhibitors open the proteasome gate (they do not), but rather, we asked what structural elements are involved in gate opening? This was the rationale of using the 20SOG.

As to the "artificiality of the system tested", we agree that the OG form is somewhat artificial. However, it may not be entirely so; deletion of the 8 amino acids may simply bypass the requirement for gate opening. We note that only 20SOG, not the WT Mtb 20S has demonstrable peptide degradation activity in vitro (Lin, Mol Microbiol 2006). Furthermore, in a most recent work reporting the in vitro reconstitution of Mtb proteasome activity towards the Pup-GFP fusion, the Mpa-mediated proteolysis by Mtb proteasome was efficient only in the open gate form of the Mtb proteasome (Striebel et al., EMBO J 2010).

There is clearly more to it than our current understanding of the gate opening process of the WT Mtb 20S in vivo. We were cautious in interpretation of our results. However, we believe our observation is significant enough to be described here in the manuscript. We added in the discussion section the new reference on the in vitro reconstitution of the Mtb proteasome activity with Mtb 20SOG, which further enhance the functional relevance of the Mtb 20SOG (Striebel et al., EMBO J 2010).

6. As I understand it, the authors suggest that the Mtb proteasome B-subunit propeptides "stick out", rather than into the catalytic core, but eventually these push their way into the core for processing. The authors further suggest that this is unique to Mtb and does not occur in the other bacterial proteasomes; however, has there been a similarly thorough analysis of proteasomes from related bacteria to suggest this process does not happen in these species? Please clarify. Along those lines p 15, Discussion - the authors state that the Mtb B-propeptide is a barrier to formation of full proteasome - why? Do the authors have any insight, are other factors involved, is this regulatory? Do the authors feel this happens in intact Mtb?

We have toned down and removed the " uniqueness" of Mtb proteasome throughout the manuscript. In revised text, we simply explain how Mtb proteasome matures and how its gate is closed, thus avoid contrasting Mtb 20S with that of other bacterial species. Per the third reviewer's suggestion, we have now noted in the revised Discussion section the potential similarities between Mtb and Rhodococcus 20S assembly.

We state the propeptide is barrier rather than promoter of the Mtb proteasome, because our previous study demonstrated that when expressed in E. coli, higher than room temperature (37 ∞C) was required to produce mature, fully assembled Mtb proteasome, and deletion of beta-propeptide alleviated the inhibitory effect and facilitated Mtb proteasome production in E. coli at room temperature (Lin et al., Mol Microbiol 2006).

We have deleted the speculative discussion (the entire third paragraph in Discussion section) on potential regulatory mechanism of the Mtb 20S maturation, per the third reviewer's suggestion.

7. p 10, section 3 - please state the purpose of using T1A mutation and how it would inhibit maturation of proteasomes; although obvious to some readers, it might not be to all.

Added in revision: "mutation at the active site prevents -propeptide removal by the The-1 mediated autoproteolysis [Groll 2003, JMB]."

8. It should be more clearly defined as to what the "anterior chamber" is relative to a particular propeptide; also it is not clear where a peptide "ascends" as described here.

Added in the revision: "the antechamber, a space between the - and the -ring". (page 10).

9. The observation that the alpha-subunits form an exceptionally tight gate is significant, and suggests that a unique mechanism may be required to activate/open it for polypeptide delivery. It was mentioned that Mpa does not robustly or stably interact with the Mtb 20S, suggesting it does not behave like a canonical eukaryotic or Archaeal proteasomal ATPase. Perhaps there should be some brief discussion about the presence of a HbYX motif in Mpa that would presumably be necessary and sufficient to activate the Mt 20S, yet does not.

Added in revision (Page 16): "On the other hand, the Mtb proteasome ATPase Mpa contains the conserved carboxyl terminal HbYX motif, particularly the penultimate Try, that has been shown to be required for the in vivo proteasome-mediated protein degradation [Darwin, 2005]. Therefore, it remains to be investigated if the Mtb 20S proteasome utilize a distinct mechanism for gate opening."

10. Discussion: page 16: the second paragraph must be omitted. Mtb never grows outside of a mammalian host in nature (the authors implicated an environmental stage of Mtb-this never happens), thus one should presume that the proteasomes are synthesized and assembled at 37 degrees. To compare Listeria to Mtb is not appropriate, as Listeria is a true environmental organism that can also grow at a wide range of temperatures, whereas Mtb cannot.

This paragraph has been removed in revision.

11. P. 16, last paragraph: The second sentence seems a bit strong, I do not believe that other prokaryotic proteasomes were presumed to be ungated, just maybe less tightly (I might be wrong). I suggest that the authors simply state that the other proteasomes are not as tightly gated as the Mtb proteasome.

Modified as suggested: "All previously characterized prokaryotic 20S proteasomes are thought to either lack a gating mechanism or loosely gated due to a partial closure of the gate".

12. Last sentence of the discussion: I didn't understand what this meant. Please clarify and rephrase.

Revised to "Such notion is also consistent with the recent finding that the 20S proteasomes have the potential to keep entire substrates in store for continual degradation, which presumably requires considerable structural plasticity of the proteasome to house the folded substrate protein (Sharon et al, 2006)."

Minor points:
13. Rhodococcus should always be in italics.

Done throughout the text.

14. p. 10: theB-propeptide needs to be separated.

Thanks for catching this typo.

15. The sections should not be numbered.

The section numbers have been deleted in revision.

16. I would omit the word "Unique" from the title.

"Unique" has been removed from the title, and also removed throughout the text. Therefore, the revised manuscript focuses on describing Mtb proteasome structures.

We wish to take this opportunity to thank the reviewer for his/her extensive comments and numerous corrections that have improved our manuscript.

Referee #2 (Remarks to the Author):

We thank the reviewer for his/her appreciation of our work. Following are point-to-point responses to the comments.

Specific comments:
In Figure 1, the authors assigned the density below the beta-ring to the beta propeptide. Although all the arguments supporting this assignment are very likely to be true, there is no direct evidence. Further, in the raw images, the propeptide seems very flexible. Considering that there is a large conformational change in the beta subunit from half proteasome to the IM-I and IM-II states, and the structure of the half proteasome is still relative low, there is a possibility that these densities are contributed by other factors, such as some part of the beta-subunit that was exposed at this conformation. Since having propeptide pointing outwards is one of the important findings of this manuscript, it is necessary to verify this assignment by, for example, using antibody or Fab that against the propeptide or using N-terminal conjugation with a GFP, or any other suitable methods.

The separate density is unlikely from part of beta-subunit other than the propeptide. The beta subunit is able to complete folding on itself, without the requirement of assembly of the mature proteasome. This is shown in the E coli expressed Mtb beta-subunit only structure (PDB ID 2JAY), which is essentially the same as the beta subunit in the mature proteasome.

We are not aware of the existence of any antibody that targets the propeptide region of beta-subunit. Producing such new antibody is outside the scope of this study. Fusion with GFP can be easily performed, but in this particular case, the fusion approach will not be able to distinguish the propeptide location: Regardless of the actual location of beta-propeptide, the GFP will be located outside, due to the limited space inside the half proteasome not being able to accommodate seven GFP that are fused to the seven beta-subunits.

Because the propeptide is small, only 56 residues, it is expected that it's footprint is weak in 2D average images or 3D map. But we have carefully validated the location of the propeptide via image processing: Refinement with 3D models in which the outside densities have been computationally removed consistently resulted in re-appearance of these densities. The densities assigned to the propeptides in 3D cryo-EM map are indeed weak, and disappear when the display threshold is raised to only 1.4 . This is noted now in the revised figure 1 legend.

We now provide in revised supplementary Fig 1 the negatively stained WT half 20S images in panel (C). The propeptide density below the beta-ring is also visible in some of the averaged side views. The same is true in the mutant T1A half proteasome (Suppl. 3).

Related to the IM-I state of Mtb proteasome, Figure 2A showed a number of class averages representing different intermediate states between half (A1) and matured (A3) proteasome. It seems that there is a range of different intermediate states. But it is also possible that these slightly different class averages are different views of the same intermediate state. The 3D reconstruction of Fig.2 B and C was calculated by arresting particles in IM-I state homogeneously with inhibitor. Although at low resolution the 3D reconstruction looks similar with the class averages shown in Fig. 2A, it is not clear if the 3D reconstruction of Figure 2B truly represents an averaged intermediate state, or is deformed by inhibitor? Further, if the structure shown in Fig. 2C is just one of a serious intermediate states shown in Fig 2A2? The authors should address this issue.

Thanks for raising this interesting issue. We agree with the reviewer that Fig. 2C is likely only one of a series of possible intermediates, because the assembly/maturation is a dynamic process. The inhibitor-treated sample formed a metastable structure. We believe that this inhibitor-arrested structure represents a true assembly intermediate because, as stated in the text, the 2D averages are indistinguishable from the natural intermediate obtained via incubation without inhibitors. To clarify, we added in the revised text (page 9): "We note that 20S IM-I might represent only one of a series of possible assembly intermediates."

The structure of IM-I is bit surprising. It is hard to think how in this conformation, where the beta-subunit of two half proteasomes are aligned and facing each other, could avoid steric clash of the protruding beta-propeptide. It is necessary to prove that this IM-I is indeed an intermediate state. One possible approach is to examine by negative stain EM the ratio of particles between IM-I and matured proteasome during the assembly process. One should find more IM-I particles in the beginning and fewer in the late of the process.

This is exactly what we mean: because of the steric clash of the beta-propeptides, in this intermediate, the propeptides should have been pushed to the interior of the chamber. We actually believe that formation of such 7-fold symmetry axis shifted intermediate is intimately related to the steric clash of propeptides in the two apposing half proteasomes ñ the intermediate forms either to facilitate propeptide translocation or as a result of the asynchronous propeptide translocation.

As we stated in the text, the inhibitor-treated intermediate is a metastable structure. As for the in vitro assembly mixture obtained by incubation without inhibitor, we have already done what the referee suggested: we observed the starting structures as nearly 100% half proteasome, and at 37 ∞C 3 hr, all mature proteasomes, but at lower temperature incubation, we observed a mixture of species that can be roughly sorted into three classes (half 20S, 20S IM-I, mature 20S). Does this 20S IM (without inhibitor) correspond to inhibitor-arrested version? We believe so, based on the similarity of structural features in 2D averages. Does the 20S IM-I contain certain level of conformational heterogeneity? Absolutely. This conformational heterogeneity is in fact reflected in the very low resolution (25 ≈) of the 3D map that we can produce. Our key point here is that there is a distinct assembly intermediate between half and mature 20S. Such intermediate was not observed in any other previously characterized proteasome systems; at least has not been reported. We interpret the formation of such novel intermediate as the result of the exterior location of beta-propeptide in the Mtb half 20S.

The side view of class average of T1A mutant half proteasome (Supplement Fig3C) looks quite different from the pai shaped low temperature cultured half proteasome (Fig1A). And it is hard to see if the propeptide is out of the half proteasome as shown in the Figure 1A. If it goes through the

same assembly process, intermediate states should also be seen. Can author comment on this? How does T1A mutant look like, if the authors culture it at low temperature?

Fig. 1A is cryo-EM images, and Suppl Fig.3C is negative stain EM. Because of different contrasting mechanism, they don't look exactly the same. New images of negatively stained WT half 20S particles have been included in revision (new Supplementary Fig 1C) to show the similarity between the WT and T1A mutant half 20S. The apparent dissimilarity between negatively stained and cryo-EM images is perhaps best illustrated by the Mtb 20S IM-I (the exactly same sample, frozen in ice as shown in Fig. 2B1 and B2, as compared with particles embedded in stain shown in supplementary Fig. 2).

We agree with the reviewer that the propeptide density is weak. This is expected because the propeptide is only 56 residues long, and most likely is unfolded at this half 20S stage. However its presence and its outside location are unambiguous, as we have shown consistently in the stained and in the iceñembedded averaged side views, and in the 3D reconstruction.

Minor points:

Applying a 2-fold symmetry in determining the 3D cryo-EM map of the Mtb 20S IM-I is still questionable. The side views of class averages do not have obvious 2-fold symmetry. In the 3D reconstruction, the half-proteasome part also lost its original 7-fold symmetry (Fig. 2C1). The authors should test if a 3D reconstruction without 2-fold symmetry indeed showing 2-fold symmetry, before applying it.

Per reviewer's suggestion, we have performed new refinement and 3D reconstruction without imposing symmetry. The resulting 3D map appears to be 2-fold symmetrical, and there is no significant difference between the asymmetrical and 2-fold symmetrical reconstructions at the resolution level achieved (25 ≈). We have added in revision: "reconstruction without symmetry resulted in a structure highly similar to the 2-fold symmetrical reconstruction".

As the reviewer correctly pointed out, most side view 2D averages don't have obvious 2-fold symmetry. There is because the Mtb 20S IM-I particle as a whole has lost the 7-fold symmetry, so there is only a single 2-fold axis relating the two half 20S. Only in the rare occasion when this single 2-fold axis is lined up with the electron beam can one observe the symmetry. In fact, the 4th panel in Fig 2B2 (cryo-EM), and the 4th panel in supplementary Fig. 2B (stained) are nearly two-fold symmetrical. This situation is very different from the mature 20S proteasome: because of the 7-fold axis along the length, there are seven 2-fold axes perpendicular to the 7-fold axis, and as such virtually all side views of the mature proteasome are nearly 2-fold symmetrical.

In Fig. 1A, the densities pointed by red arrows are very weak, almost at the level of noise background. Maybe variance of this region will show clearer density of the propeptide?

In addition to the cryo-EM 2D averages shown in Fig. 1A, we now include in revision the negatively stained 2D averages of the WT Mtb half 20S in revised supplementary Fig. 1C.

Suggest removing the word "unique" from the title.

The word "unique" has been removed from the title in revision.

Page 12, correct 20S IM-I to 20S IM-II

Corrected. Thanks.

Page 15, correct Fig.5E and F to Fig.5D and E.

Corrected.

Page 20, Figure legend G and F to G and H

Corrected. We thank the reviewer for his insightful comments and numerous suggestions that have

markedly improved our manuscript.

Referee #3 (Remarks to the Author):

The report by Li and colleagues reports a number of novel EM and X-ray structures for the Mycobacterium tuberculosis 20S proteasome and its assembly intermediates. These structures reveal 1) unusual positioning and changes in position of the 56-residue beta propeptide that seem to correlate with effects on assembly; 2) interesting dynamic changes in subunit conformation and relative positioning between the mature 20S proteasome and its precursors; and 3) novel structural features of the alpha-ring gate that allows a tight seal of the pore, at least in the precursor-like form called Mtb 20S IM-II.

Overall, I feel these structures provide substantial insight into the structural diversity of 20S proteasomes and the states they can go through in the process of assembly. The novel features of this work make it suitable for publication in the EMBO Journal. The paper is generally well written, with clear figures, and the data appear to be solid, although I am not a structural biologist. The one complaint I have is the unnecessary attempt to cast the Mtb 20S as extremely different from all previously studied 20S proteasomes. I think earlier work on the Rhodococcus and Thermoplasma proteasomes suggests otherwise. I would like to see these similarities represented more accurately in the final version of the paper, as suggested below.

The authors continually suggest that the Mtb beta propeptide has a uniquely inhibitory effect on proteasome assembly. I think the similarities to the other well studied actinomycete proteasome, that from Rhodococcus, may be greater than the differences. First, it should be noted that in the Zuhl et al. (1997) reference, it was found that the Rhodococcus beta propeptide supplied in trans allowed far more rapid dimerization of half proteasomes compared to normal beta subunits with the propeptide in cis, consistent with the idea that the propeptide has to get repositioned or processed for stable dimerization to occur. This is reminiscent of the negative effect on dimerization suggested here. This likely similarity of the two actinomycete proteasomes should be noted. Second, the propeptide structure in preholoproteasome or 20S IM-II intermediate here is, as the authors point out, similar to the structure seen with the analogous intermediate of the Rhodococcus proteasome. Although the idea that the temperature block to assembly of the Mtb proteasome is related to the function of the proteasome in the M. tuberculosis life cycle, the comparable temperature shift has not been done with Rhodococcus, to my knowledge.

The reviewer's point is well taken. We have removed the "uniqueness" argument throughout the manuscript, and also removed the word "unique" from the title.

In the discussion section, first paragraph, we have removed "In all proteasomes characterized so far, the -propeptides either promote or play no essential role in assembly. This makes Mtb proteasome a mysterious exception." Per reviewer's advice, we have added (page 15): "Apparently, the Mtb - subunit is able to properly fold on itself, in the absence of the -propeptide or the -subunit, as shown by the crystal structure of the -subunit alone (PDB ID: 2JAY). In the Rhodococcus 20S, the b-propeptide helps folding of the b-subunit, and promotes the 20S assembly [Zhul et al., 1997]. However, the Rhodococcus beta propeptide when supplied in trans allowed far more rapid dimerization of half proteasomes compared to normal beta subunits with the propeptide in cis [zhul et al., 1997]. This observation is consistent with the idea that the propeptide has to get repositioned or processed for stable dimerization to occur. Such notion is reminiscent of the negative effect of b-propeptide on dimerization of Mtb half 20S. Therefore, despite the existence of individual characteristics of the proteasomes, certain crucial steps in the assembly process might be shared by the two actinomycete proteasomes. Further support to the notion is provided by the highly similar structures of the Rhodococcus preholoproteasome and its counterpart in Mtb (the Mtb 20S IM II), particularly at the ordered propeptide region (Supplementary Fig. 4B)."

Similarly, the authors state that archaeal proteasomes have a partially open gate or are not gated, but I think this view is dated. The Rabl et al. (2008) paper, for example, shows clear electron density in the pore region of the T. acidophilum 20S proteasome, and there is definitely gating behavior of this proteasome through PAN binding. The slightly open gate might have more to do with how the proteasomes are isolated and purified than real differences in gating properties. At most, the

difference is one of degree.

This statement has been deleted in revision.

The experimental details are a little thin. For example, culture volumes or grams of wet cell pellets that were harvested should be given, as should protein concentrations, buffer volumes and column sizes. In general, the reader should be able to know how to replicate exactly what was done in the paper without having to contact the authors.

More details have been added to the experimental procedure in the supplementary material. We thank the reviewer for this advice and critique.

