THE EMBO JOURNAL

Manuscript EMBO-2009-72460

Interactions of PAN's C-termini with archaeal 20S proteasome and implications for the eukaryotic proteasome-ATPase interactions

Yadong Yu, David Smith, Homin Kim, Victor Rodriguez, Alfred Goldberg, Yifan Cheng

Corresponding author: Yifan Cheng, University of California San Francisco

Review timeline:

Submission date:	05 September 2009
Editorial Decision:	01 October 2009
Revision received:	25 November 2009
Acceptance letter:	26 November 2009
Accepted:	27 November 2009

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 October 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by four reviewers with expertise in proteasome biology as well as crystal structure and cryo-electronmicroscopy. As you will see from their comments attached below, a majority of these referees considers your new detailed molecular insight into proteasome docking/activation via the C-termini of PAN, using a hybrid activator complex, of interest and would thus in principle support publication in The EMBO Journal pending adequate revision of a number of specific points. The main issues needing to be addressed are on one hand connected to the discussion and (over)interpretation of obtained results, but also include some control experiments as raised in points 1 and 2 of referee 1 and point 1 of referee 2.

Should you be able to adequately address these various issues, we should be happy to consider a revised manuscript for publication. I would therefore like to invite you to prepare such a revision in the spirit of the reviewers' comments and suggestions. Please be however reminded that it is EMBO Journal policy to allow a single round of major revision only, and that it is therefore essential that you diligently answer to all the points raised at this stage if you wish the manuscript ultimately to be accepted. 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 your revision.

Yours sincerely,

Editor
The EMBO Journal

_____

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The authors report the cryo-EM and crystal structures of the archaeal 20S proteasome core particle (CP) with a hybrid ATPase activator containing the C-termini of PAN and the core of PA26 docked to the ends of the CP. Activation of the 20S was previously demonstrated by this group using a combined structural and biochemical approach with HbYX-motif peptides. The current study's major contribution to our understanding of 20S activation by PAN (and by extrapolation, eukaryotic regulatory particles) is atomic detail of the PAN C-terminal tail in direct contact with residues in the 20S alpha-ring intersubunit "pockets," which are responsible for gate-opening and substrate entry into the eukaryotic 20S. The authors also attempted to discern the intersubunit pocket specificity of the eukaryotic ATPase subunit C-termini (expressed as fusions to PAN) for the specific intersubunit pockets found in the eukaryotic alpha ring by mutationally recreating them in the archaeal alpha subunit ring. Although this approach is clever, the authors were able to reconstitute and purify 20S particles containing only two of seven intersubunit pockets (only one of which was activated by any of the PAN hybrids). Thus, little new information about ATPase specificity for specific intersubunit pockets was provided, making it difficult to convincingly interpret the outcome of their biochemical analysis.

This is the first atomic-resolution view of the important HbYX motif interaction with the alpha pocket, albeit in an artificial context, allowing details of binding and gate-opening to be inferred.After the changes suggested below, which will require re-review, I think the paper is suitable for publication in the EMBO Journal.

1. The replacement of the C-terminus of PA26 with that of PAN was an excellent approach. The fact that it activates the PA26 version with an activation loop mutation suggests it is working in the way the HbYX sequence would in its natural context. However, there is no actual test of this assumption. One additional mutant that should be tested for activation and binding is the Y-to-F mutation in the HbYX motif of the context of the PA26 mutant to show that this no longer activates. This is important because the symmetry matched PA26-20S complex might not require all the sequence elements normally required within the HbYX of specific Rpt subunits.

2. The experiments with specific eukaryotic alpha-pocket mimics built into the T. acidophilum alpha subunit are a little contrived, at least by themselves (see above). The experiment would be significantly improved if the authors were to mutate the binding pocket of alpha3-alpha4 in yeast and show that mutating residues that seem to be crucial for binding/opening via Rpt5 based on the X-ray structure are indeed important in the natural context. As it stands, the data in Fig. 5 really do not tell us more than we already knew from the crosslinking analysis of Gillette et al. (2008).

3. The paper is generally well written, but there will need to be some editing of the English.

4. In Figure 1 legend, please indicate the full coloring scheme for panels A and B.

5. In Figure 1D legend, please correct "xx µg" with the proper amount of 20S.

Referee #2 (Remarks to the Author):

The importance of the 26S proteasome in cellular regulation is well-documented. The 26S proteasome is composed of two subcomplexes, a core particle (CP, also termed as 20S proteasome) and one or two the proteasome activator known as 19S regulatory particles (RP). The RP recognizes the polyubiquitinated substrates, unfolds the substrate proteins, activates the CP, and translocates the unfolded substrates into the interior of the CP. The present study by Yu and coworkers determined

the structure of the key elements of the RP and describes how the RP activates the CP. The authors previously identified the Hb-Y-X motif in the RP ATPases as a key for the gate-opening of the CP. By using cryo-EM analysis, the authors showed the Hb-Y-X motif are inserted into inter-subunit pockets of the alpha ring of the CP and stimulates conformational change for the gate opening. However, the previous study failed to determine the structure of the Hb-Y-X motif and the detailed mechanism how this motif induces the gate-opening remained unclear. In the present study, the authors create a chimera protein that consists of PA26, another proteasome activator, and the Hb-Y-X motif of PAN, archaea proteasomal ATPase. The PA26-PAN chimera protein efficiently binds and activates the CP. The authors determine the structure of the chimera-CP by cryo-EM and by X-ray crystallography. Consistent with their previous study, the alpha subunits rotated around the central channel of the CP, suggesting that the Hb-Y-X motif in the chimera induces the conformational change of the CP. Importantly, the specific interactions between the Hb-Y-X motif and the CP pockets are determined; Hb residue-hydrophobic patch, Y residue-Gly19, and X residue-Lys66. Finally, the authors investigate specific subunit-subunit interactions by using six different sequences of the proteasomal ATPase subunits and the mutated CPs. Although the Hb-Y-X motif of Rpt2 does not stimulate the CP activity, that of Rpt5 specifically activates the CP with alpha3-4 pocket. This observation suggests that the specific pair between the RP subunit and the CP pocket is formed in the mature 26S proteasome.
Although the experimental design is somehow artificial, but the present study is a very interesting and important study for our understanding of how the RP activates the CP. This reviewer has only minor comments, mainly concerning discussion and mistypes as noted below.

Points
1. Previous studies suggest that two of six Rpt subunits, Rpt2 and Rpt5, can stimulate the gate-opening of mammal 20S proteasome (Smith et al., 2007. and Gillette et al., 2008). In the paper by Gillette et al., Rpt2 and Rpt5 has additive effect for the CP activation, suggesting that Rpt2 and Rpt5 can bind with the CP simultaneously. In the present study, only PAN-Rpt2 chimera can stimulate wild-type T20S activity and only PAN-Rpt5 chimera can activate alpha3-4 20S proteasome (Figure 5). Is this a structural problem using mutant or nature properties of PAN? I think the author would confirm the activity of the chimeras using mammal or yeast 20S proteasomes. Also, the authors would discuss the above notion.

2. Baumeister's group recently reported ATPase order using cryo-EM map and bioinformatics (Forster et al, 2009, BBRC). In their model, Rpt2 and Rpt5 associate with alpha3-4 and alpha4-5, respectively. The authors would comment on the difference.

3. Mistypes and others
-In page 4, correct "P26" to "PA26".
-In material and methods, second paragraph, correct "300 NaCl" to "300 mM NaCl".
-In figure legend 1B, is the amino acid residue indicated by stick E102?
-In figure legend 1D, insert the 20S amount.

Referee #3 (Remarks to the Author):

The active sites of the proteasome are sequestered in an internal chamber and substrate accessibility, the rate limiting step in proteolysis, is performed by proteasomal regulators. In this study, Yu et al. show the atomic details of how the conserved C-terminal HbYX-motif of PAN interacts with the intersubunit pockets of the 20S proteasome and suggest possible ideas for gate opening. The authors create a hybrid proteasomal activator based on the heptameric PA26 molecule, in which the activation loop was eliminated by site-directed mutagenesis to disable PA26's ability to induce gate opening.

The performed experiments are solid; however, the interpretation of the results looks like to be exaggerated. It is discussed that PAN forms a hexameric ring, whereas PA26 is heptameric. Thus the hybrid is a nice tool to get further insights in understanding the HbYX interactions, but it is too speculative to discuss about proteasomal docking mechanism of PAN and even the eukaryotic Base-complex based on the proteasome-PA26 hybrid results; just to emphasize, PA26 and PAN differ completely in architecture, assembly and function and currently there are no high resolution data available on the proteasome-PAN complex. As has been shown for PA26, there are more than one

interaction sites with the proteasome. Definitely, the HbYX-motif plays an important role in PAN-proteasome complex formation, as shown by the authors, but these results do not allow any proposals on the asymmetry between seven- and six-fold regulator bindings including allostery.

Furthermore, Yu et al. suggest a conformational change in the 20S alpha-ring, which was not seen in any crystal structure including the 20S-proteasome-PA26-hybrid-complexes, which the authors have shown to be similar to that of wild type. This referee is surprised about the suggestion of a conformational change in the alpha-rings, which was expected as an induced fit mechanism, since it is even not observed in their crystal structure. The authors indicate that based on EM each alpha-subunit rotate about 7{degree sign} pivoting around the central channel, but this would be in disagreement with an RMSD of only 2.5A by structural superposition of alpha-rings between 20S proteasome-hybrid complex and 20S proteasome.

As indicated, so far there exists no crystal structure showing large structural rearrangements in the proteasomal alpha-ring, thus the suggestion of an induced fit mechanism might be adventurous, but for this referee still an interesting idea. Introduction and Discussion are in some parts redundant, thus the manuscript can be shortened. This referee suggests to publish the work of Yu et al. in EMBO-Journal after minor revisions.

Referee #4 (Remarks to the Author):

The manuscript by Yu et al uses a combination of high-resolution cryo-EM, X-ray crystallography and mutagenesis to characterise the interaction between the C-termini of the regulatory PAN ATPase and the archaeal proteosome. This is an interesting topic. The work seems to be generally well performed.

MAJOR CORCERNS

A major concern is that this work does not provide sufficiently significant and novel biological insights beyond the results recently provided by the same authors (Rabl et al. Mol Cell May 2008):

- The interaction between the C-terminal motif of PAN and the proteosome was already well described in their MolCell paper.

- The cryo-EM structure shows a very good resolution and the image processing seems to be well performed. But the structure provides very little biological information that was not already available in the previous work by the authors (Rabl et al. Mol Cell May 2008). The cryo-EM reconstruction appears to be mostly a control experiment supporting that the hybrid PA26/PAN regulator behaves as PAN.

- The major novelty of this work is the crystal structure of the C-terminal motif of PAN bound to the proteosome, but I have some doubts that the new information provided by this structure justifies the whole paper. An expert in the proteosome field should judge this.

- Being the X-ray structure the main advance in this manuscript, the resolution of the structure is relatively poor (4A resolution). The refinement statistics are somehow strange: the B-factors are high; the R factors are low for 4A resolution, etc. Since the refinement yields so unexpected good statistics for a poor resolution, the authors should provide a more detailed description of the methods used.

MINOR COMMENTS:

- The quality of the figures could be improved. Sometimes is hard to follow the comments on the text in the figures. The conformational changes in the cryo-EM structure described in the text are hard to see in Figure 2.

- The use of a hybrid PA26/PAN activator is very clever and I think it provides a valuable tool. But, I have some concern about the physiological relevance of a 7-fold interaction given that PAN is a hexamer.

- The resolution of the cryoEM structure might be slightly over-estimated when using the 0.143 criterion. A 0.5 cross-correlation of the FSC could provide a more realistic estimate.

- More details about the image processing for cryo-EM would be helpful. For instance, what was the initial template used for refinement?

- There is a certain confusion in Figure 2. The text related to this figure has a full paragraph describing the "pseudo-atomic" model built using several structures. On the other hand, the legend indicates that the atomic structure solved in this manuscript was fitted within the map. This should be clarified.

- Much of the discussion is dedicated to eukaryotic proteosomes when the results obtained in this manuscript deal with the archaeal proteins.

1st Revision - authors' response 25 November 2009

Referee #1 (Remarks to the Author):

The authors report the cryo-EM and crystal structures of the archaeal 20S proteasome core particle (CP) with a hybrid ATPase activator containing the C-termini of PAN and the core of PA26 docked to the ends of the CP. Activation of the 20S was previously demonstrated by this group using a combined structural and biochemical approach with HbYX-motif peptides. The current study's major contribution to our understanding of 20S activation by PAN (and by extrapolation, eukaryotic regulatory particles) is atomic detail of the PAN C-terminal tail in direct contact with residues in the 20S alpha-ring intersubunit "pockets," which are responsible for gate-opening and substrate entry into the eukaryotic 20S. The authors also attempted to discern the intersubunit pocket specificity of the eukaryotic ATPase subunit C-termini (expressed as fusions to PAN) for the specific intersubunit pockets found in the eukaryotic alpha ring by mutationally recreating them in the archaeal alpha subunit ring. Although this approach is clever, the authors were able to reconstitute and purify 20S particles containing only two of seven intersubunit pockets (only one of which was activated by any of the PAN hybrids). Thus, little new information about ATPase specificity for specific intersubunit pockets was provided, making it difficult to convincingly interpret the outcome of their biochemical analysis.

This is the first atomic-resolution view of the important HbYX motif interaction with the alpha pocket, albeit in an artificial context, allowing details of binding and gate-opening to be inferred. After the changes suggested below, which will require re-review, I think the paper is suitable for publication in the EMBO Journal.

1. The replacement of the C-terminus of PA26 with that of PAN was an excellent approach. The fact that it activates the PA26 version with an activation loop mutation suggests it is working in the way the HbYX sequence would in its natural context. However, there is no actual test of this assumption. One additional mutant that should be tested for activation and binding is the Y-to-F mutation in the HbYX motif of the context of the PA26 mutant to show that this no longer activates. This is important because the symmetry matched PA26-20S complex might not require all the sequence elements normally required within the HbYX of specific Rpt subunits.

Response: We agree and appreciate this suggestion. Based on this reviewerís comment we have now made a Y-to-F mutation in the penultimate residue of the PA26E102A-PAN9 activator. Indeed, this hybrid activator with a Y-to-F mutation in its C-terminal HbYX motif does not activate the 20S CP, nor did we observe any complex between this activator and the 20S by negative staining EM. This result is consistent with the requirement for the HbYX motif in the PAN ATPase for 20S gate opening. Therefore, it indeed confirms that the PA26 hybrid activator used for our structural study induces gate opening like PAN, via an HbYX-dependent mechanism. We have now included this

new data in the manuscript as Table 1. We added a paragraph in page 9 to describe this result.

2. The experiments with specific eukaryotic alpha-pocket mimics built into the T. acidophilum alpha subunit are a little contrived, at least by themselves (see above). The experiment would be significantly improved if the authors were to mutate the binding pocket of alpha3-alpha4 in yeast and show that mutating residues that seem to be crucial for binding/opening via Rpt5 based on the X-ray structure are indeed important in the natural context.

Response: We agree and appreciate this suggestion. We initially did not plan to take this mutagenesis approach in the current study, due to the concern that when residues critical for the Rpt5 binding are mutated, it may seriously damage the functions of the entire 26S proteasome. That is why we took a different approach to create an artificial 20S that mimics the intersubunit pocket. We have now started a systematic mutagenesis screening of residues within the intersubunit pocket between alpha 3 and 4. Our current study suggested that the HbYX motif interacts with a number of residues within the intersubunit pocket. We are therefore screening single as well as multiple residue mutations. However, it will require a very significant effort to complete this screen, which is beyond the scope of the current manuscript. In addition, the goals and conclusions from those experiments are ancillary to the major theme and conclusions of this manuscript. We therefore prefer to complete those experiment and publish them separately.

As it stands, the data in Fig. 5 really do not tell us more than we already knew from the cross-linking analysis of Gillette et al. (2008).

Response: We respectfully disagree. The cross-linking analysis of Gillette et al. (2008) showed that Rpt5 is cross-linked to 4, but does not indicate which 4 containing pocket. Thus, there are two possible intersubunit pockets that Rpt5 can bind to, i.e. 3-4 or 4-5. Our data presented in Figure 6, suggests that Rpt5 binds to the 3-4 pocket, thus 3-4 pocket is favored over the 4-5 pocket. This is consistent with the data of Gillette et al, but further narrows the results down to one favored pocket on the left side of the 4. Therefore, though these conclusions are ancillary to the main point of our manuscript, they do give us new and useful information regarding the specific interaction of the Rptís with the 20S intersubunit pockets, which is a major unsolved problem.

3. The paper is generally well written, but there will need to be some editing of the English.

Response: We appreciate reviewerís comment and we have given the manuscript to several colleagues to help us correct the typographical and grammatical errors in the manuscript.

4. In Figure 1 legend, please indicate the full coloring scheme for panels A and B.

Response: We added the following sentences in the figure legend of Figure 1A to explain the color scheme used: "Two neighboring -subunits are colored in light and dark green. The PA26 subunit that binds to this intersubunit pocket is colored in blue." For Figure 1B, we added the word "orange" to indicate the color of PA26 activation loop and C-terminus.

5. In Figure 1D legend, please correct "xx µg" with the proper amount of 20S.

Response: We apologize for the typographical error. It is now corrected to the right number.

Referee #2 (Remarks to the Author):

The importance of the 26S proteasome in cellular regulation is well-documented. The 26S proteasome is composed of two subcomplexes, a core particle (CP, also termed as 20S proteasome) and one or two the proteasome activator known as 19S regulatory particles (RP). The RP recognizes the polyubiquitinated substrates, unfolds the substrate proteins, activates the CP, and translocates the unfolded substrates into the interior of the CP. The present study by Yu and coworkers determined the structure of the key elements of the RP and describes how the RP activates the CP. The authors previously identified the Hb-Y-X motif in the RP ATPases as a key for the gate-opening of the CP. By using cryo-EM analysis, the authors showed the Hb-Y-X motif are inserted into inter-subunit pockets of the alpha ring of the CP and stimulates conformational change

for the gate opening. However, the previous study failed to determine the structure of the Hb-Y-X motif and the detailed mechanism how this motif induces the gate opening remained unclear. In the present study, the authors create a chimera protein that consists of PA26, another proteasome activator, and the Hb-Y-X motif of PAN, archaea proteasomal ATPase. The PA26-PAN chimera protein efficiently binds and activates the CP. The authors determine the structure of the chimera-CP by cryo-EM and by X-ray crystallography. Consistent with their previous study, the alpha subunits rotated around the central channel of the CP, suggesting that the Hb-Y-X motif in the chimera induces the conformational change of the CP. Importantly, the specific interactions between the Hb-Y-X motif and the CP pockets are determined; Hb residue-hydrophobic patch, Y residue-Gly19, and X residue-Lys66. Finally, the authors investigate specific subunit-subunit interactions by using six different sequences of the proteasomal ATPase subunits and the mutated CPs. Although the Hb-Y-X motif of Rpt2 does not stimulate the CP activity, that of Rpt5 specifically activates the CP with alpha3-4 pocket. This observation suggests that the specific pair between the RP subunit and the CP pocket is formed in the mature 26S proteasome.

Although the experimental design is somehow artificial, but the present study is a very interesting and important study for our understanding of how the RP activates the CP. This reviewer has only minor comments, mainly concerning discussion and mistypes as noted below.

Points

1. Previous studies suggest that two of six Rpt subunits, Rpt2 and Rpt5, can stimulate the gate opening of mammal 20S proteasome (Smith et al., 2007. and Gillette et al., 2008). In the paper by Gillette et al., Rpt2 and Rpt5 has additive effect for the CP activation, suggesting that Rpt2 and Rpt5 can bind with the CP simultaneously. In the present study, only PAN-Rpt2 chimera can stimulate wild-type T20S activity and only PAN-Rpt5 chimera can activate alpha3-4 20S proteasome (Figure 5). Is this a structural problem using mutant or nature properties of PAN? I think the author would confirm the activity of the chimeras using mammal or yeast 20S proteasomes. Also, the authors would discuss the above notion.

Response: There is a structural reason why PAN-Rpt5 and PAN-Rpt3 do not activate wild type archaeal 20S proteasome, but only PAN-Rpt2 does. The hydrophobic residues in the HbYX motif of yeast Rpt5 and Rpt3 are both Phe, which has a larger side chain than the Leu residue of PANís C-terminal HbYX motif. Most likely, Phe makes it harder to bind to the archaeal 20S intersubunit pocket. Even though Phe is a hydrophobic residue, our previous work (Table 2 of Smith, 2007) showed that a L-to-F mutation in PANís HbYX motif significantly reduced the stimulation of gate opening, to only 20% of the wild type PAN. In contrast, Rpt2 has an HbYX motif that is very similar to that in PANís C-terminus, making it easier for Rpt2 to bind to the wild type archaeal 20S than Rpt5 and Rpt3. Therefore, it is reasonable that only PAN-Rpt2 chimera can stimulate the wild-type archaeal 20S, but not other PAN-Rpt chimeras. As for the alpha3-4 20S proteasome, we changed many residues within the intersubunit pocket (Supplement Table 2, and supplement Figure 3) in the hope that it would mimic the yeast alpha3-4 intersubunit pocket and it would accommodate C-terminus of only one specific Rpt subunit. This approach turned out to be successful.

As suggested by the reviewer, we also performed an experiment to test the activities of the PAN-Rpt hybrid on yeast 20S. However, none of these activators, including the wild type full length PAN, stimulate the yeast 20S gate opening to any significant level. This is structurally understandable. Although one of the C-termini of PAN-Rpt5 or PAN-Rpt2 should be able to bind to one of the seven intersubunit pockets in the yeast 20S, the rest of the five C-termini, which all have the same length, cannot bind to any other pocket and they may allosterically reduce the binding affinity of the only binding pocket. The fact that the PAN-Rpt2 and PAN-Rpt5 each activates a different 20S confirms their activity.

The Figure attached here included the results from this experiment. However, we feel this result do not add any new information, and we prefer not to include it to the manuscript.

2. Baumeister's group recently reported ATPase order using cryo-EM map and bioinformatics (Forster et al, 2009, BBRC). In their model, Rpt2 and Rpt5 associate with alpha3-4 and alpha4-5, respectively. The authors would comment on the difference.

Response: Based on information combined from structural, biochemical and bioinformatic studies,

Forster et al. proposed a model in which the order of Rpt-subunits is 1-2-6-3-4-5, clockwise viewed from distal end. This is one of the two possible orders of the Rpt subunits that satisfy structural and newly identified Rpt-subcomplex constrains. Furthermore, their model suggested that Rpt2 binds to the 3-4, and Rpt5 binds to 6-7. In the present study, our data suggests Rpt5, instead of Rpt2, binds to 3-4. Also, the Rpt5 can be cross-linked to 4 and Rpt2 can be cross-linked to 7 (Gillette et al. 2008). We therefore favor our model that Rpt5 binds to the pocket between 3-4 and Rpt2 binds to the pocket between 6-7. We now added this into the Discussion.

3. Mistypes and others
-In page 4, correct "P26" to "PA26".
-In material and methods, second paragraph, correct "300 NaCl" to "300 mM NaCl".
-In figure legend 1B, is the amino acid residue indicated by stick E102?
-In figure legend 1D, insert the 20S amount.

Response: These typographical errors are now corrected.

Referee #3 (Remarks to the Author):

The active sites of the proteasome are sequestered in an internal chamber and substrate accessibility, the rate limiting step in proteolysis, is performed by proteasomal regulators. In this study, Yu et al. show the atomic details of how the conserved C-terminal HbYX-motif of PAN interacts with the intersubunit pockets of the 20S proteasome and suggest possible ideas for gate opening. The authors create a hybrid proteasomal activator based on the heptameric PA26 molecule, in which the activation loop was eliminated by site-directed mutagenesis to disable PA26's ability to induce gate opening.

The performed experiments are solid; however, the interpretation of the results looks like to be exaggerated. It is discussed that PAN forms a hexameric ring, whereas PA26 is heptameric. Thus the hybrid is a nice tool to get further insights in understanding the HbYX interactions, but it is too speculative to discuss about proteasomal docking mechanism of PAN and even the eukaryotic Base-complex based on the proteasome-PA26 hybrid results; just to emphasize, PA26 and PAN differ completely in architecture, assembly and function and currently there are no high resolution data available on the proteasome-PAN complex. As has been shown for PA26, there are more than one interaction sites with the proteasome. Definitely, the HbYX-motif plays an important role in PAN-proteasome complex formation, as shown by the authors, but these results do not allow any proposals on the asymmetry between seven- and six-fold regulator bindings including allostery.

Response: We agree that our current work presented in this manuscript does not address the issue of symmetry mismatch between PAN and 20S. However, we respectfully disagree with this reviewerís comment that it is too speculative to discuss the docking mechanism of the extreme C-terminus of PAN. Both gate opening activities (data presented in Figure 1D) as well as conformational changes (newly added Figure 5, see below, and Supplement Figure 2) revealed by our structure indicate that the hybrid activator opens the gate in the same fashion as PANís C-terminal peptides alone. Moreover, the Y to F mutation data (see above) has also confirmed that our hybrid activator induces 20S gate opening in an HbYX-dependent manner, like the proteasomal ATPases.

Furthermore, Yu et al. suggest a conformational change in the 20S alpha-ring, which was not seen in any crystal structure including the 20S-proteasome-PA26-hybrid-complexes, which the authors have shown to be similar to that of wild type. This referee is surprised about the suggestion of a conformational change in the alpha-rings, which was expected as an induced fit mechanism, since it is even not observed in their crystal structure. The authors indicate that based on EM each alpha-subunit rotate about 7{degree sign} pivoting around the central channel, but this would be in disagreement with an RMSD of only 2.5A by structural superposition of alpha-rings between 20S proteasome-hybrid complex and 20S proteasome.

Response: We thank this reviewer for pointing out the small value of RMSD. The rotation of the subunit we observed is hinged around helix located in the wedge between the neighboring subunits. We aligned three structures together, our 20S-PA26/PAN (3ipm), wild-type 20S-PA26 (1ya7) and wild-type 20S (1pma), on their -rings. This hinge helix (residue 81-104) has smallest RMSD of

only 0.7 Å (3IPM-1YA7) and 0.7 Å (3ipm-1pma). The distal helix (residue 168-180) has a largest RMSD of 3.3 Å (3ipm -1ya7) and 3.2 Å (3ipm-1pma). The Ca atom of Arg178 moved 4.1 Å from 1pma to 3ipm, and moved 5.2 Å from 1ya7 to 3ipm. The Pro 17 moved about 1.8 Å from 1pma to 3ipm and only 0.9 from 1ya7 to 1pma. The RMSD of 2.5 Å is an average of all Ca atoms of subunit, and is misleadingly small. We now added a paragraph to describe these changes and included a figure (Figure 5) so that the conformational change is better described. We also added the similar figure to describe the difference between 3ipm and 1ya7 as Supplement Figure 2.

As indicated, so far there exists no crystal structure showing large structural rearrangements in the proteasomal alpha-ring, thus the suggestion of an induced fit mechanism might be adventurous, but for this referee still an interesting idea. Introduction and Discussion are in some parts redundant, thus the manuscript can be shortened. This referee suggests to publish the work of Yu et al. in EMBO-Journal after minor revisions.

Response: We appreciate the suggestion of this reviewer to publish our manuscript in the EMBO Journal. We now added a Figure 5 to illustrate the conformational change in the subunit, and marked the distance the distal helix moved as well as the reverse turn loop moved upon binding of our hybrid activator. We hope that our response above as well as the added figure can convince this reviewer about the conformational change revealed by both the single particle cryoEM structure as well as by the crystal structure presented in our manuscript.

During the revision of this manuscript, a paper was published by Chris Hillís laboratory (Stadtmueller et al. 2009, JBC, C109.070425), which presented three crystal structures of 20S-PA26 variants. One of these structures has the PA26ís C-terminus replaced with the sequence of PAN. This is an approach similar as ours presented here, and indeed this structure does not show any conformational change in the 20S. However, there are a number of noticeable and significant differences between studies presented by this JBC paper and ours. One of such differences is that its PA26 variant with PANís C-terminus still carries an intact activation loop, while our hybrid complex has the activation disabled so that it impairs the binding of PA26 to the 20S. Also, its PA26 variants do not show dependence of HbYX motif that a V-to-F mutation of penultimate residue did not impair its capability of binding and inducing gate opening of 20S, while our hybrid showed a clear dependence of HbYX motif demonstrated by an experiment suggested by reviewer #1 (above). Another difference is that these PA26 variants have their C-termini directly replaced by PAN, while we inserted two glycine residues in between as a flexible linker. The most important difference is that the conformation of 20S in this newly published study is essentially the same as the wild-type 20S-PA26, while ours clearly shows a conformational change (Figure 5 and supplement figure 2). Although it is not entirely clear if the differences in the hybrid construct mentioned above contribute to such structural difference, we do favor such thoughts. It certainly requires additional carefully designed experiments to fully address such structural difference. Since this paper was submitted and published during the revision of our manuscript, we believe it is beyond the scope of our current manuscript to discuss such differences and their causes. However, if reviewers recommend us to do so, we can add above discussions to the manuscript.

Referee #4 (Remarks to the Author):

The manuscript by Yu et al uses a combination of high-resolution cryo-EM, X-ray crystallography and mutagenesis to characterise the interaction between the C-termini of the regulatory PAN ATPase and the archaeal proteosome. This is an interesting topic. The work seems to be generally well performed.

MAJOR CORCERNS

A major concern is that this work does not provide sufficiently significant and novel biological insights beyond the results recently provided by the same authors (Rabl et al. Mol Cell May 2008):

- The interaction between the C-terminal motif of PAN and the proteosome was already well described in their MolCell paper.

Response: While this reviewer is correct, that this structural study itself (like many other structural

studies in general) does not provide novel biological insights, but it certainly provides new high-resolution understanding of the structural basis for proteasome gate opening by the associated ATPases and goes far beyond our previous work (Rabl, 2008, Mol Cell). That paper only demonstrated where the C-termini of PAN bind and what globe conformational change such binding induces. In our current work, we presented the atomic details of this critical interaction and the resulting conformational changes in the subunit leading to gate opening.

- The cryo-EM structure shows a very good resolution and the image processing seems to be well performed. But the structure provides very little biological information that was not already available in the previous work by the authors (Rabl et al. Mol Cell May 2008). The cryo-EM reconstruction appears to be mostly a control experiment supporting that the hybrid PA26/PAN regulator behaves as PAN.

Response: The purpose of generating a hybrid activator was to facilitate the crystal structure determination. It was therefore very important to validate if the hybrid activator indeed behaves as we designed, that it activates the 20S in an HbYX-dependent manner, like PAN, in solution. Single particle cryoEM is the best approach to validate this critical assumption, and we believe that this data further corroborates the conformational changes that we observed in the crystal structure. Therefore, indeed, the cryoEM work included in this manuscript serves as a necessary control experiment that is important to show. And this data serves as an important validation to the crystal structure.

- The major novelty of this work is the crystal structure of the C-terminal motif of PAN bound to the proteosome, but I have some doubts that the new information provided by this structure justifies the whole paper. An expert in the proteasome field should judge this.

Response: We appreciate this reviewerís concern. We believe that the positive and confirming comments from other reviewers on this point support our judgment and can resolve this concern. Also, knowing these detailed mechanisms are also important for elucidation of related question, e.g. how ATP-binding, as well as ubiquitin conjugates (see recent papers from labs of Kloetzel, DeMartino and Goldberg) and hydrolysis influence gate opening.

- Being the X-ray structure the main advance in this manuscript, the resolution of the structure is relatively poor (4A resolution). The refinement statistics are somehow strange: the B-factors are high; the R factors are low for 4A resolution, etc. Since the refinement yields so unexpected good statistics for a poor resolution, the authors should provide a more detailed description of the methods used.

Response: The refinement followed standard crystal structure refinement procedures, with a 7-fold non-crystallographic symmetry imposed throughout the iterative model building and refinement. The density map and the omit map indeed show features better than a usual 4A map. We believe such good quality maps, as well as the good statistics, are because of the high quality phase information used in the molecular replacement. The template model used was the wild-type 20S-PA26 complex which has an overall architecture very similar to that of our complex. The high B-factor could be caused by the factor that we did not have sufficient high-resolution information for a Wilson-plot, thus our calculation of the B-factor may not be accurate. We now included this information in the Methods part of the manuscript.

MINOR COMMENTS:

- The quality of the figures could be improved. Sometimes is hard to follow the comments on the text in the figures. The conformational changes in the cryo-EM structure described in the text are hard to see in Figure 2.

Response: We appreciate this suggestion, and added a new Supplementary Figure 1C to show the conformational change revealed by the single particle cryoEM reconstruction.

- The use of a hybrid PA26/PAN activator is very clever and I think it provides a valuable tool. But, I have some concern about the physiological relevance of a 7-fold interaction given that PAN is a hexamer.

Response: Because we shared the same concern of this reviewer, we performed various control experiments described above and deleted the activation loop of PA26 to insure that the properties of the fusion resemble those of PAN. One argument supporting the importance of our studies and conclusion is the report in press by Hill and co-workers discussed above in response to reviewer #3. Their study also addressed the structure of PANís C-termini in a similar approach, but did not remove the PA26ís activation loop and did not observe rotation of the alpha-subunits. The existence of two studies emphasizes the interest and importance of the question and the novelty of our approach and results.

- *The resolution of the cryoEM structure might be slightly over-estimated when using the 0.143 criterion. A 0.5 cross-correlation of the FSC could provide a more realistic estimate.*

Response: We appreciate this reviewerís point. Both 0.5 and 0.143 cross-correlation criterions are now marked in the supplementary Figure 1.

- *More details about the image processing for cryo-EM would be helpful. For instance, what was the initial template used for refinement?*

Response: We now included more detailed description about the image processing in the Material and Method section.

- *There is a certain confusion in Figure 2. The text related to this figure has a full paragraph describing the "pseudo-atomic" model built using several structures. On the other hand, the legend indicates that the atomic structure solved in this manuscript was fitted within the map. This should be clarified.*

Response: We thank this reviewer for pointing out this. We now correct it and stated clearly in the figure legend.

- *Much of the discussion is dedicated to eukaryotic proteasomes when the results obtained in this manuscript deal with the archaeal proteins.*

Response: Based on our crystal structure, we designed a number of mutants that mimic that intersubunit pockets and PAN mutants that mimic the yeast Rpt subunits. Our experiments, although performed on archaeal protein, does provide new insights to the yeast proteasome.

Acceptance letter 26 November 2009

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. I have now carefully looked through your responses to the various points raised initially by the reviewers, and overall find that you have adequately and diligently addressed the main issues. I have also noted the recent publication of the competing manuscript you mentioned, and therefore feel that rapid publication of your more comprehensive analysis is highly warranted. We shall therefore be happy to accept your manuscript for publication in The EMBO Journal.

