THE EMBO JOURNAL

Manuscript EMBO-2009-72985

A Novel ATP-dependent Conformation in p97 N-D1 Fragment Revealed by Crystal Structures of Disease Related Mutants

Wai Kwan Tang, Dongyang Li, Choi-chi Li, Lothar Esser, Renming Dai, Liang Guo and Di Xia

Corresponding author: Di Xia, National Cancer Institute, NIH

Review timeline:

Submission date:	28 October 2009
Editorial Decision:	23 December 2009
Revision received:	05 March 2010
Editorial Decision:	31 March 2010
Revision received:	20 April 2010
Accepted:	26 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 23 December 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. I apologise for the delay in returning with a decision on your manuscript, but after several discussions, unfortunately the final referee will not be able to return his/her report and I have decided to take a decision based on the two referee reports that I have received, which I copy below. As you will see from their comments the referees in general find the study interesting. However, Referee #1 finds that the study is difficult to interpret in the absence of the structure of WT-ATPgS structure and states that this data is necessary. I have discussed this matter with the Executive Editor who also agrees that this data should be included in the current study for publication in the EMBO Journal. Given the overall interest if you are able to address these issues we would be willing to consider a revised version of the manuscript.

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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):
Tang and colleagues report on the structures of three p97-ND1 fragments, two bearing common IBMPFD mutations (R155H and R95G) and one with a introduced mutation expected to have similar effect as the natural mutations (R86A) bound to ADP and/or ATPgS. Using X-ray crystallography, the authors determine the structures of p97-ND1 mutants R155H (with ATPgS and ADP), R95G (ATPgS), R86A (ATPgS) and R155H (ADP). Most strikingly, while it has been suggested by previous cryo-EM (Rouiller et al., 2002; Beuron et al., 2003) and SAXS studies (Davies et al., 2005), the study is the first report using X-ray crystallography to show the N domain in a position which is not in the same plane as the D1 domain. This study brings high resolution information on the conformational differences between the ADP and ATPgS states, such as a coiled to helix transition in the linker domain between the N- and D1- domain. However since, this study is the first reported study showing ATPgS bound to any of the AAA domains of p97 (previous studies were done with AMP-PNP), it is not clear if the different positioning of the N domain is due to the IBMPFD mutations or to ATPgS.

Major critique:
1- It is not clear if the non-planar position of the N domain is due to the IBMPFD mutations (as implied by the title) or by binding of ATPgS. According to the X-ray scattering experiments presented in the paper (which shows a similar reduction in radii of gyration for all p97 ND1 domains, wild-type and mutants), it seems unlikely that the apical position of the N domain is due to the mutations, but it is not proven either that it is due to the ATPgS. They suggest that the fact that they were able to crystallize ND1 with ATPgS is because of the IBMPFD mutations (page 14, second paragraph; and page 17: "Because the use of mutant p97 appears to be critical to the observed conformational changes in the N-domain"). However, since the authors report a similar affinity for WT ND1 for ADP and ATPgS and they name a paper in preparation (Tang Tang WK, Li DY, Esser L, Xia D (2009) Crystallization of the human AAA+ protein p97 in the presence of ATPgS In preparation), this suggests that it is also possible to crystallize WT p97 with ATPgS, and that they have indeed done so. More importantly, this suggests that the authors have the answer to the fundamental questions as to 1) whether the non-planar conformation of the N domain is because of the different nucleotide or the mutations, and 2) how the mutations affect the difference in nucleotide affinity observed. As the target of the paper seems to be the IBMPFD mutants, I feel that this information should be included in the present manuscript.

2- Similarly, the authors observe that in the ND1-R155H-ADP, the N-domain adopts the down conformation characteristic of WT with ADP in D1. However, they do not describe if there are any differences between the two structures at high resolution that could explain the difference of affinity between the WT and the mutants.

Minor critiques:
1- The previous study by SAXS (Davies et al., Structure 2005) which position the N domain at different position is not cited.
2- Similarly, in the introduction, the authors say that: "structural investigations using various approaches invariably found ADP bound in the D1-domains and the N-domain adopted the in-plane conformation,... see review by Pye at al, 2006". This sentence should be reworded as part of the structural investigations; cryo-EM and SAXS studies did not always find the N-domain in the in-plane conformation.
3- For clarification purposes, especially in the title of the results section, the authors should refer to p97 N-D1 and not simply to p97 as this falsely infer that they are referring to the full length protein.
4- The radii of gyration measured by SAXS for mutant ND1 increases from 55-56 Å in with ADP to 52-53 Å with ATPgS, while the diameter of the mutant ND1 ring determined by X-ray crystallography expands from 162 Å to 164 Å. This difference should be explained.
5- In the Material and Methods, the authors refer to a paper in preparation to describe: "Protein expression and purification of wild type and mutant p97 N-D1 fragments is detailed elsewhere (Tang et al., 2009)". A paper in preparation is not a good reference to use to describe methods.

Referee #3 (Remarks to the Author):

The authors examine the effects of naturally occurring mutations in p97 using structural and biochemical analysis of the N-D1 fragment. The IBMPFD mutation sites largely occur in the N-D1 interface, and the authors find that these changes lower the affinity for ADP and allow more facile exchange of ATPgS (whose affinity increases concomitantly with the decrease of ADP affinity). The authors obtained crystal structures of two IBMPFD mutants and find that the N domain position is "up" rather than coplanar as seen in previous structures of N-D1 and full-length wild-type p97. This finding is strengthened by a third mutation designed by the authors that is not associated with disease but is also in the N-D1 interface; the effect is similar. First, there is a coil-helix transition in the N-D1 linker that is proposed to couple ATP binding to changes in position of the domain. Second, the conserved N-terminal peptide of the N domain is partially ordered, and forms interactions with both D1 and the linker in the ATP bound state. These are the first high-resolution molecular data that address the coupling between ATP binding and the position of the N domain, as has been inferred from a variety of studies of this and other AAA proteins. Overall the work is technically excellent and the data look very solid.

The obvious concern with this study is that all of the analysis has been done on the N-D1 fragment. The model (Fig. 5) presumes that the nucleotide-dependent transitions taking place in the N-D1 construct will be identical with that of full-length p97. It is known that the D2 domain is the essential ATPase in this system, and previous structural data (e.m., crystallography and SAXS) have shown coupling of the nucleotide state of D2 to changes in the N domain. It has been shown that exchange of the bound D1 nucleotide is inhibited by D2, and the linker between D1 and D2 appears to couple the nucleotide binding sites of the two subunits. Furthermore, interactions between D1 and D2 rings have been shown to be essential in the mechanisms of other AAA ATPases (Costa et al., 2006 & Glynn et al., 2009). Thus, it seems an inappropriate extrapolation of data from the N-D1 fragment to statements about the full-length mechanism as illustrated in figure 5.

Similarly, the paper concludes (p 21) "...the conformational change in the N-domain...is important for the control of D1 ATPase activity". However, the data say nothing about the control of D1 ATPase activity in the real enzyme. It seems equally, if not more, likely that D2 controls this. The fact that the N domain conformation is coupled to the D1 ATPase activity is a necessary consequence of the allostery in this system regardless of what is controlling the ATPase activity.

The ITC data presented here, as well as previous work (Briggs et al., 2008) indicate asymmetry in the nucleotide binding properties of p97, but the model presented in Fig. 5 does not explain nor account for this. Asymmetry in nucleotide-binding and hydrolysis has been implicated in the mechanisms of other AAA+ ATPases but the model presented ignores this.

Other specific questions/concerns.

Page 8, 2nd paragraph: It would be useful to define the changes in N more precisely; what is the rotation of the N-domain. If the D1 domains in the ATPgS and ADP states are superimposed, what are the rotational transformations that relate the N domains (e.g. spherical polar angles)?
p14, first paragraph. "In the ATP-bound state," should read "In the ATPgammaS-bound state,"

Page 16, last line: it is incorrect to say that nucleotide-dependent changes in the N-domain have not been observed previously; in fact, this was an explicit point of the Davies et al 2005 SAXS reference as well as the em studies. In some cases the N density was lost, consistent with disordering; to this reviewer, these are still changes in the N domain even if they are not structurally well defined.

On page 21, first paragraph, the authors need to be more careful in their terminology of "empty" or "locked" states. Essentially these refer to partial or full occupancy of sites within the hexamer. It is unclear if a fully saturated hexamer ever exists physiologically.

Page 24: Were NCS restraints imposed in any of the refinements? This should be stated in the methods.

Page 25-26: Was there really no salt in the buffers used for SAXS and ITC?

Page 26: Although the affinity measurement is robust, determination of binding stoichiometry is extremely sensitive to the concentrations used in the ITC data analysis. The authors used a particular molar extinction coefficient for determination of concentration. With only a single Ni-NTA purification step, it is not clear that they have accounted accurately for nucleotide that copurified with the protein since there is no explicit exchange step performed. Can they verify that they have no nucleotide bound at the start of the experiment? Given the significant absorption at 280 nM of adenine nucleotides, if there is some partial occupancy of the nucleotide in the extinction coefficient used may be inaccurate and could influence the n value obtained in the experiment.

1st Revision - authors' response 05 March 2010

Comments from the Editor:

As you will see from their comments the referees in general find the study interesting. However, Referee #1 finds that the study is difficult to interpret in the absence of the structure of WT-ATPgS structure and states that this data is necessary. I have discussed this matter with the Executive Editor who also agrees that this data should be included in the current study for publication in the EMBO Journal. Given the overall interest if you are able to address these issues we would be willing to consider a revised version of the manuscript.

>As addressed in our response to the referee #1's comments (see below), we apologize for our oversight in the use of an inappropriate title for a reference, which apparently led to the misunderstanding that we had already achieved solving the wild type N-D1 p97 structure with ATPγS bound. The correct title for that reference is "Purification, crystallization and preliminary X-ray diffraction analysis of disease-related mutants of p97", which has been published in Acta Crsytallographica Section F [1]. In that short communication, we detailed conditions on crystallizing the three p97 N-D1 mutants in the presence of ATPγS.

Whether and how p97 undergoes nucleotide dependent N-domain conformational change has been a controversial issue in mechanistic studies of this protein. Our ability to show the dramatic conformational change in the N-domain of IBMPFD mutants of p97 in the presence of ATPγS or ADP demonstrates for the first time at submolecular level that such a change indeed takes place. Although we are not able to provide a crystal structure of the wild-type p97 N-D1 fragment with bound ATPγS, our SAXS experiments clearly demonstrated that the wild type p97 is able to undergo similar nucleotide-dependent conformational change in solution.

So, why didn't the wild type p97 crystallize in the ATPγS bound state? It is not due to the lack of effort on our part. Our lab has been working on this for more than five years; we had several false positives where crystals of wild type p97 N-D1 in the presence of ATPγS turned out to have ADP bound at the D1 domain. Similar efforts in other laboratories have also failed. Our ITC experiments provided clues for this question. They showed that (1) there are more pre-bound ADP molecules in the D1 domain of the wild type p97, (2) mutants have a lowered ADP binding affinity towards the D1 nucleotide binding site, and (3) pre-bound ADP in the D1 domain can be replaced by ATPγS in mutant but not in wild type. The likely explanation for failure in crystallizing wild type p97 with bound ATPγS is the asymmetric distribution of the pre-bound ADP in hexamerically arranged protomers. Because ADP in these subunits of a wild type p97 are not exchangeable, only empty sites will bind ATPγS in solution and undergo conformational change. Consequently, there will be both ADP and ATPγS bound subunits in hexamers in the presence of ATPγS, which are non-uniform in N-domain conformation and difficult to crystallize. The case for the IBMPFD mutants is different,

because they have a lowered binding affinity for ADP, which can be exchanged in the presence of ATPγS, resulting in a uniform protein preparation suitable for crystallization. This result also implies that subunits of a wild type p97 hexamer most likely work asymmetrically. It also suggests that in order to exchange the pre-bound ADP some forms of modification to the affinity of the D1 domain for ADP must take place. This is consistent with one referee's comments that such a modification may come from the ATP hydrolysis from the D2 domains or the binding of various adaptor proteins.

We believe that our work has contributed to the field of AAA protein in general and p97 in particular in our understanding of the mechanism of nucleotide coupled conformational change in AAA proteins and how interruption of this mechanism might lead to dysfunction of these proteins. Another interesting result of our work is that we found in our ATPγS structures the bound Mg2+ ions at the active site, which is the first time they were observed in any of the p97 crystal structures.

Referee #1:
Major critique:
1- It is not clear if the non-planar position of the N domain is due to the IBMPFD mutations (as implied by the title) or by binding of ATPgS. According to the X-ray scattering experiments presented in the paper (which shows a similar reduction in radii of gyration for all p97 ND1 domains, wild-type and mutants), it seems unlikely that the apical position of the N domain is due to the mutations, but it is not proven either that it is due to the ATPgS. They suggest that the fact that they were able to crystallize ND1 with ATPgS is because of the IBMPFD mutations (page 14, second paragraph; and page 17: "Because the use of mutant p97 appears to be critical to the observed conformational changes in the N-domain"). However, since the authors report a similar affinity for WT ND1 for ADP and ATPgS and they name a paper in preparation (Tang Tang WK, Li DY, Esser L, Xia D (2009) Crystallization of the human AAA+ protein p97 in the presence of ATPgS In preparation), this suggests that it is also possible to crystallize WT p97 with ATPgS, and that they have indeed done so. More importantly, this suggests that the authors have the answer to the fundamental questions as to 1) whether the nonplanar conformation of the N domain is because of the different nucleotide or the mutations, and 2) how the mutations affect the difference in nucleotide affinity observed. As the target of the paper seems to be the IBMPFD mutants, I feel that this information should be included in the present manuscript.

>We thank the reviewer for the overall positive comments on our work.

We apologize for our oversight in the use of an inappropriate title for a reference, which apparently led to the misunderstanding that we had already achieved solving the WT ND1 p97 structure with ATPγS-bound. The correct title for that reference is "Purification, crystallization and preliminary X-ray diffraction analysis of disease-related mutants of p97", which was published in Acta Crystallographica Section F [1]. In that short communication, we detailed conditions on crystallizing the three p97 N-D1 mutants in the presence of ATPγS. The revised manuscript has the title for that reference corrected.

Whether and how p97 undergoes nucleotide dependent N-domain conformational change has been a controversial issue in mechanistic studies of this protein. Our ability to show the dramatic conformational change in the N-domain of IBMPFD mutants of p97 in the presence of ATPγS or ADP demonstrates for the first time at submolecular level that such a change indeed takes place. Although we are not able to provide a crystal structure of the wild-type p97 N-D1 fragment with bound ATPγS, our SAXS experiments clearly demonstrated that the wild type p97 is able to undergo a similar nucleotide-dependent conformational change in solution.

With respect to the first question above, our crystal structures showed unequivocally that the conformational change in the N-domain is indeed a nucleotide dependent event for the IBMPFD mutant p97. The question is whether the N-domains of the wild type p97 undergo the same nucleotide-dependent conformational change. Because crystals of the wild type p97 N-D1 with bound ATPγS have so far been impossible to obtain, we used small angle X-ray scattering (SAXS) to detect possible conformational changes of the wild type p97 in solutions in response to the presence of different nucleotides. The experimental result is strongly in favor of the wild type

protein undergoing similar conformational change. So, why didn't wild type p97 N-D1 crystallize in the presence of ATPγS? Our ITC data suggest that the presence of non-exchangeable pre-bound ADP in D1 domains of the wild type p97 hexamers may introduce asymmetry to the Ndomain conformation when the proteins are exposed to ATPγS, leading to a nonuniform conformation of p97 hexamers in solution. Therefore, in order to crystallize the wild type p97 in the presence of ATPγS, all its six N-domains in a hexamer, not just a few individual subunits, must undergo a coordinated nucleotide dependent change, which is not possible. Conceivably, by modifying the affinity of the D1 domain for ADP, as demonstrated in our current work, the IBMPFD mutations are able to do just that by reducing the binding affinity of D1 domain for ADP, leading to complete exchange of ADP with ATPγS and successful crystallization. For the wild type p97, we think that such a coordinated movement might be a rare event, as asymmetry in N-domain conformation as well as in D1 nucleotide binding states in hexameric p97 seem to be important in p97 function.

With respect to the effect of mutations to the binding of various nucleotides at D1 domain, we found by using ITC that IBMPFD mutations reduced the affinity of ADP to the D1 domain (Table 4 in the submitted manuscript), while the affinity for ATPγS remains basically unchanged, when considering the amount of pre-bound ADP. More importantly, pre-bound ADP in the D1 domain in mutant p97 was found to be exchangeable with ATPγS. This conclusion was obtained after considering the amount of pre-bound ADP in both wild type and mutants during ITC titration and the observation of biphasic titration profile for the mutants. Taken together, the observations made by ITC lead to the following conclusions: (1) Pre-bound ADP in the D1 domain of IBMPFD mutant p97 is no longer non-exchangeable, leading to the biphasic titration curve in the ITC experiment with the first phase being the titration of empty sites and the second one being the titration of ADP pre-bound sites. (2) An asymmetric p97 hexamer with different nucleotides bound at the D1 domain may be important for p97 function and deficiencies in IBMPFD mutant p97 may be due to the loss of this asymmetry. In the discussion of the revised manuscript, we have reiterated these points.

2- Similarly, the authors observe that in the ND1-R155H-ADP, the N-domain adopts the down conformation characteristic of WT with ADP in D1. However, they do not describe if there are any differences between the two structures at high resolution that could explain the difference of affinity between the WT and the mutants.

> The Cα traces of the two structures (wild type and R155H mutant with ADP bound) were superimposable with an rmsd of 1.03 Å over 433 residues (Table S1 of the submitted manuscript). The environments of the nucleotide-binding pockets for both structures were carefully examined and no significant difference was found. Because both N-D1 structures with ADP-bound were solved at 2.9 Å [2] and 3.42 Å, respectively, and in two different space groups, we conclude that this rmsd does not represent a significant change in structure. Hence, we think that a likely explanation for the apparent difference in ADP binding lies in the fact that the N-D1 interface for mutant p97 has been changed, leading to changes in the electrostatic environment of the ADP binding pocket because the interface is only a thin layer of amino acid residues from the ADP binding site. In the revised manuscript we added a sentence to reflect this point (Page 10).

Minor critiques:
1- The previous study by SAXS (Davies et al., Structure 2005) which position the N domain at different position is not cited.

> In the revision, we cited this reference in the discussion to reflect one of its conclusions on the conformational change of the N-domain (Page 18).

2- Similarly, in the introduction, the authors say that:
"structural investigations using various approaches invariably found ADP bound in the D1-domains and the N-domain adopted the in-plane conformation,... see review by Pye at al, 2006". This sentence should be reworded as part of the structural investigations; cryo-EM and SAXS studies did not always find the N-domain in the in-plane conformation.

> In the revision, we have changed the wording from "structural investigation" to "crystallographic investigation" to specifically emphasize that in all crystal structures

determined to date, wild type p97 invariably has bound ADP in the D1-domain (Page 4).

3- For clarification purposes, especially in the title of the results section, the authors should refer to p97 N-D1 and not simply to p97 as this falsely infer that they are referring to the full length protein.

> Following the reviewer's suggestion, we have changed the title to "A novel ATPdependent conformation in p97 N-D1 fragment revealed by crystal structures of disease related mutants"

4- The radii of gyration measured by SAXS for mutant ND1 increases from 55-56 Å; in with ADP to 52-53 Å; with ATPgS, while the diameter of the mutant ND1 ring determined by X-ray crystallography expands from 162 Å; to 164 Å;. This difference should be explained.

> The definition of the radius of gyration (*R*g) tells how the mass and density of the molecule is distributed. That is how close the mass is centered versus the axis of rotation. The diameter of the hexameric ring is the real space measurement on the size of the macromolecule. Therefore, there is no correlation between the values of *R*g and diameter, meaning two molecules may have identical diameters but very different radii of gyration. The SAXS experiments relate measured scattering intensity distribution to *R*g of the molecule in solution.

In Table 3 of our submitted manuscript, we report the observed changes in *R*g upon exposure of p97 N-D1 to different nucleotide; such changes are consistent for both wild type and IBMPFD mutants. This change reflects a redistribution of mass in the molecule and is consistent with the observed N-domain conformational change. In the submitted manuscript, we had a sentence on page 11 "The smaller *R*g values indicate the presence of more spherical molecules in solution for the N-D1 in the ATPγS form.", which explains the difference between the *R*g and diameter of a molecule.

5- In the Material and Methods, the authors refer to a paper in preparation to describe: "Protein expression and purification of wild type and mutant p97 N-D1 fragments is detailed elsewhere (Tang et al., 2009)". A paper in preparation is not a good reference to use to describe methods.

> While this manuscript was under review, the paper in reference was accepted and published in Acta Crystallography Section F [1]. As mentioned above, the title of that reference was inappropriate and changed to "Purification, crystallization and preliminary X-ray diffraction analysis of disease-related mutants of p97". In the revision, this reference has been updated and readers can refer to that article for more detailed information about the expression, purification and crystallization procedures.

Referee #3

1-The obvious concern with this study is that all of the analysis has been done on the N-D1 fragment. The model (Fig. 5) presumes that the nucleotide-dependent transitions taking place in the N-D1 construct will be identical with that of full-length p97. It is known that the D2 domain is the essential ATPase in this system, and previous structural data (e.m., crystallography and SAXS) have shown coupling of the nucleotide state of D2 to changes in the N domain. It has been shown that exchange of the bound D1 nucleotide is inhibited by D2, and the linker between D1 and D2 appears to couple the nucleotide binding sites of the two subunits. Furthermore, interactions between D1 and D2 rings have been shown to be essential in the mechanisms of other AAA ATPases (Costa et al., 2006 & Glynn et al., 2009). Thus, it seems an inappropriate extrapolation of data from the N-D1 fragment to statements about the full-length mechanism as illustrated in figure 5.

> We thank the reviewer for the positive comments and constructive suggestions.

We agree with the reviewer that the three domains (N, D1 and D2) in p97 need to communicate with each other for p97 to function properly. Conceivably, interactions exist not only vertically between D1 and D2 rings but also laterally between individual protomers. In fact, in our submitted manuscript, we touched upon one possible role of D2 in regard to the N-domain conformational movement as well as nucleotide binding state in D1. We speculated that, in the wild type scenario, external stimuli such as ATP

hydrolysis in D2 or adaptor protein binding to the N-domain may be needed to trigger the exchange of pre-occupied ADP in D1, resulting in the movement of the N-domains. It is also possible that the ATP binding or hydrolysis in a neighboring subunit may trigger nucleotide exchange in D1 in a sequential model. Unfortunately, it is difficult to present these ideas or speculations in a graphical form in Figure 5, as those do not easily translate into some forms of physical motions that can be portrayed. We are sorry if the Figure 5 in our submitted manuscript cast the impression that D1 and D2 act independently; it was not the effect intended. In the revised version, we have added one paragraph of discussion on the possible role of D2 domain in controlling N-domain conformation and also emphasized in the figure legend the potential interactions existing between D1 and D2 domains.

We also agree with the reviewer that structural studies have provided evidence for coupling of observed structural changes with specific nucleotides present in various experimental setups. Specific changes in N-domain conformations in response to the presence of various nucleotides were reported by EM and SAXS studies. Furthermore, genetic and biochemical experiments in p97 and in other type II AAA proteins such as the *E. coli* ClpA and ClpB, as well as the yeast Hsp104 indicated a communication existing between the two rings. However, among the crystallographically determined full-length p97 structures in the presence of various nucleotides, the coupling of the Ndomains' conformational change to the nucleotide states in D2 has not been observed, because D1 domains in ALL these structures are bound with ADP and N-domains are ALL in the Down conformation. One possibility is that although both D1 and D2 can bind to specific nucleotide in solution, the extents of binding in the two domains is different, leading to asymmetry in the N-domain conformation. The process of crystallization subsequently selects molecules with a specific N-domain conformation, i.e. molecules that happens to have all D1 domains in the ADP state. Based on our and others' studies, we believe that nucleotide binding states of the D1 domain may be coupled to the ATP hydrolysis in D2, not just to nucleotide binding alone. Alternatively, the binding of adaptor proteins to the N-domain may also influence the nucleotide binding state in D1. Since the exact nature of this communication is not understood and further studies are required.

In our revision, we have revised the last section extensively and changed the section title to "Implications for p97 ATPase cycle in the D1 domain" (page 22).

2-Similarly, the paper concludes (p 21) "...the conformational change in the N-domain...is important for the control of D1 ATPase activity". However, the data say nothing about the control of D1 ATPase activity in the real enzyme. It seems equally, if not more, likely that D2 controls this. The fact that the N domain conformation is coupled to the D1 ATPase activity is a necessary consequence of the allostery in this system regardless of what is controlling the ATPase activity.

> As discussed above, we are aware of all experimental data for p97 and other type II AAA proteins such as ClpA, ClpB, and yeast Hsp104 that suggest the existence of communication between the D1 and D2 rings. In fact, we did mention in our submitted manuscript that the nucleotide binding states in D1 may be influenced by ATP hydrolysis in D2. So we think we agree with the reviewer on this point. With respect to the N-domain's role in D1 nucleotide state, here is our logic. Our data demonstrated that IBMPFD mutations lower the ADP binding affinity to the D1 nucleotide binding sites. Since all IBMPFD mutations are located at the N-D1 interface, they alter the interactions between the N- and D1-domain, and consequently change the ADP binding affinity at the D1 site. We have noticed that there are a number of positively charged residues of the N-domain contributing to the N-D1 interface. Therefore, we hypothesize that by binding to some adaptor proteins, the N-domain may be able to influence the ADP binding at the D1 site. The fact that p47 binding can significantly inhibit ATPase activity of p97 seems consistent with this notion [3]. In the revision, we have added one paragraph to address this point (pages 23-24) .

3-The ITC data presented here, as well as previous work (Briggs et al., 2008) indicate asymmetry in the nucleotide binding properties of p97, but the model presented in Fig. 5 does not explain nor account for this. Asymmetry in nucleotide-binding and hydrolysis has been implicated in the

mechanisms of other AAA+ ATPases but the model presented ignores this.

> We appreciate the reviewer's insight. Indeed, we suspected the presence of asymmetry in the wild type p97 hexamers by our success in crystallizing the mutant p97 in the presence of ATPγS and our inability to crystallize wild type p97 under similar conditions. The ITC data revealed different amount of pre-bound ADP in various p97 constructs and the biphasic titration profiles for mutant p97, suggesting nonexchangeable ADP in the D1 domain of wild type p97. The presence of nonexchangeable ADP in D1 appears to be an important property of the wild type p97, leading to asymmetry in the nucleotide binding state in D1 as well as in N-domain conformation of a hexamer. More significantly, the asymmetry may have important implications for p97 function, as it eliminates the model for a concerted mechanism of p97 function. However, we must admit that the ITC data itself is not sufficient to tell what kind of asymmetry a p97 hexamer possesses, meaning that we can't determine the pattern of asymmetry to predict whether the N-domains undergo a sequential or random movement during p97 catalytic cycles. Due to these uncertainties, in our current model, we focus on N-D1 alone and assume that the state of each protomer is independent of the states of other protomers. Nevertheless, in the revision, we have revised Figure 5 to reflect the observed asymmetry for the wild type p97 and have in many places discussed the implication of the suggested asymmetry in p97 function.

Other specific questions/concerns.
4-Page 8, 2nd paragraph: It would be useful to define the changes in N more precisely; what is the rotation of the Ndomain. If the D1 domains in the ATPgS and ADP states are superimposed, what are the rotational transformations that relate the N domains (e.g. spherical polar angles)?

> As suggested by the reviewer, we determined the relative motion undergoing by the N-domain in response to different nucleotide binding states as following:

We first aligned the hexmeric p97 in ATPγS form with that in ADP form, using only the D1 domain (rmsd is 0.692 for 169 residues). We identified the residue G208 as the point of reference for our calculation as this residue is the first residue remaining fixed in position in both forms (Figure 1, point A). We then computed the centers of gravity for N-domains in both conformations (Figure1, points B and C). We found that the translational vector **T** linking the two centers of gravity is about 12.5 Å and the angle α is ,11°. By superimposing the N-domain in the ADP form (Figure 1, point B) with that in ATPγS form (Figure 1, point C) in O, we found that the transformation requires a rotation of 92.6° about an axis with polar angles of phi of 77.2° and psi of 21.4°. This information has been added to the revised manuscript and presented as Figure S3.

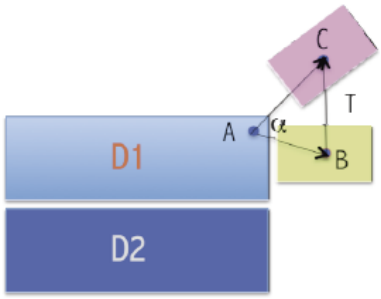

Figure 1 Calculation of the rotation and translation undergoing by the N-domain upon changes in bound nucleotide in the D1-domain.

5-p14, first paragraph. "In the ATP-bound state," should read "In the ATPgammaS-bound state,"

> The "ATP-bound state" has been changed to "ATPγS-bound state" in the revision.

6-Page 16, last line: it is incorrect to say that nucleotidedependent changes in the N-domain have not been observed previously; in fact, this was an explicit point of the Davies et al 2005 SAXS reference as well as the em studies. In some cases the N density was lost, consistent with disordering; to this reviewer, these are still changes in the N domain even if they are not

structurally well defined.

> In our revisions, we have made the correction to that statement and made it clear that conformational changes assigned to N-domains have been observed by SAXS and EM studies in response to the presence of different nucleotides in solution (page 18).

7-On page 21, first paragraph, the authors need to be more careful in their terminology of "empty" or "locked" states. Essentially these refer to partial or full occupancy of sites within the hexamer. It is unclear if a fully saturated hexamer ever exists physiologically.

> It is our observation by ITC that all IBMPFD mutants exhibit decreased affinity for ADP compared to the wild type at the D1 domain. We further observed, also by ITC, that in the wild type p97 the ADP bound at the D1 domain is difficult to exchange. We hypothesize that this high affinity, non-exchangeable ADP binding state in some subunits of the wild type p97 hexamer reflects the need of the enzyme to control the conformation of the N-domain and to maintain their asymmetry, which may be altered by ATP hydrolysis in D2 or by adaptor binding to the N-domain. To define this high affinity, non-exchangeable ADP binding state in wild type p97 protomers, we used the term “locked” state. Note that the “locked” state does not refer to a partially occupied site. This state is also the key to the asymmetry in the p97 wild type whereas in mutants, this “locked” state is in a rapid equilibrium with the ADP-open state. Also, in our proposed model, all the nucleotide states mentioned are referring to D1 only and assume that the state of each protomer is independent of the states of other promoters. In the revision, we tried to make this point clearer (Page 23).

8-Page 24: Were NCS restraints imposed in any of the refinements? This should be stated in the methods.

> In our crystallographic refinement, NCS restraints were applied throughout. Following the reviewer’s suggestion, we have amended the Materials and Methods section.

9-Page 25-26: Was there really no salt in the buffers used for SAXS and ITC?

> We didn’t add any salt when performing both SAXS and ITC experiments. This buffer condition is the same as that used for ATPase activity assay except for the type of nucleotides used.

10-Page 26: Although the affinity measurement is robust, determination of binding stoichiometry is extremely sensitive to the concentrations used in the ITC data analysis. The authors used a particular molar extinction coefficient for determination of concentration. With only a single Ni-NTA purification step, it is not clear that they have accounted accurately for nucleotide that copurified with the protein since there is no explicit exchange step performed. Can they verify that they have no nucleotide bound at the start of the experiment? Given the significant absorption at 280 nM of adenine nucleotides, if there is some partial occupancy of the nucleotide in the extinction coefficient used may be inaccurate and could influence the n value obtained in the experiment.

> After purification by Ni-NTA affinity chromatography, wild type as well as mutant p97 were concentrated and dialyzed extensively in storage buffer. Moreover, protein samples were again dialyzed against the working buffer before they were used for ITC experiments. After these extensive dialysis procedures, the amount of free nucleotide in the sample solution is negligible. However, we found by our ITC experiment that all samples contained various amounts of ADP and the pre-bound ADP can’t be removed completely by extensive dialysis alone, consistent with the previous study by Briggs et al., 2008 [4].

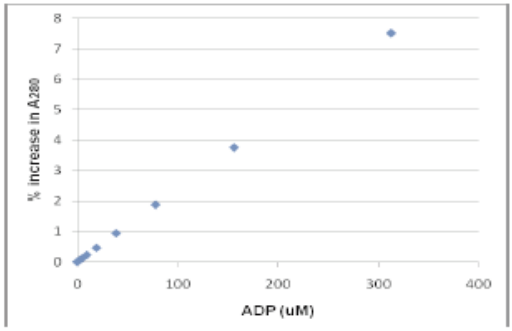

Figure 2. Relative contribution of ADP adsorption at 280 nm to that of BSA. The BSA concentration is 5 mg/ml.

We share the reviewer's concern with respect to the accuracy of the estimated amount of pre-bound ADP determined by ITC in various p97 samples, because the stoichiometry determined in ITC is tied to the protein concentration. In order to have a rough idea of the amount of errors due to protein samples containing nucleotides, we did the following experiment (Figure 2) where the percentage increase in absorbance on a BSA solution were monitored as a function of ADP concentration. The figure shows the percentage contribution to the absorbance of a 5 mg/ml BSA (72 uM) solution by ADP at 280 nm at various concentrations. Apparently, the contribution of nucleotides to the absorption at 280 nm is relatively small and depends on the amount of bound nucleotides, the composition, size and concentration of the protein. As one can clearly see, even at a molar ratio of 1:1 between BSA and ADP, the contribution is relatively small (< 2%). P97 at 10 mg/ml (185 uM) has an equivalent UV absorbance at 280 nm to that of BSA at 5 mg/ml, at which the protein concentrations of p97 preparations was often determined. Based on Figure 2, we estimated that the expect error in determining the protein concentration for p97 N-D1 should be less than 5% even for the worst case scenario, in which all D1 subunits are pre-occupied with ADP. Therefore, the partial occupancy of the nucleotide does not significantly affect the accuracy of protein concentration determination by the extinction coefficient method.

References

[1] Tang, W., Li, D., Esser, L., and Xia, D. (2009) Purification, crystallization and preliminary X-ray diffraction analysis of disease-related mutants of p97. Acta Crystallography Section F, 65:1166-1170.

[2] Zhang, X., Shaw, A., Bates, P. A., Newman, R. H., Gowen, B., Orlova, E., Gorman, M. A., Kondo, H., Dokurno, P., Lally, J., Leonard, G., Meyer, H., van Heel, M. & Freemont, P. S. (2000). Structure of the AAA ATPase p97. *Mol Cell* **6**, 1473-1484.

[3] Meyer, H. H., Kondo, H. & Warren, G. (1998). The p47 co-factor regulates the ATPase activity of the membrane fusion protein, p97. *FEBS Lett* **437**, 255-257.

[4] Briggs, L. C., Baldwin, G. S., Miyata, N., Kondo, H., Zhang, X., and Freemont, P. S. (2008) Analysis of nucleotide binding to P97 reveals the properties of a tandem AAA hexameric ATPase. *J Biol Chem* **283**, 13745-13752.

2nd Editorial Decision 31 March 2010

Your revised manuscript has been reviewed by the original referees and they recommend publication pending some minor revision.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.

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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors have adequately addressed my concerns.
In their response to the referees, the authors have extensively explained that they were not able to crystallize the WTp97 in the presence ATPgS. They have mentioned in the results section that WT was only able to crystallize with ADP and in the discussion that this conformation was resisting crystallization. They have made clear in the publication that according to their SAXS data, the wild type ND1 domain is likely to undergo similar conformational change, but that the IBMFD mutations alter the affinities of D1 for ADP.
On a minor issue, as p97 full-length and N-D1 domains have previously been solved bound with ADP, it would be nice if the authors were to mention if any differences is observed between their structure and the previously published ones.
Overall, I am in favor of publishing the manuscript.

Referee #3 (Remarks to the Author):

The authors have addressed the points made in my original review. The paper is significantly clearer and the authors have made appropriate references to the role of D2 in the full-length enzyme. The arguments for the different conformations being nucleotide state dependent are solid. The outstanding question is the relevance of the observed changes to what is happening in the full-length enzyme. Much more work will be needed to understand how the principal ATPase activity provided by D2 relates to the changes seen here. Although how changes are driven remain unclear, I believe that the paper represents a significant advance in understanding the coupling of conformational change to nucleotide state in this class of AAA proteins by defining changes in the N-D1 linker region and its effects on the position of the substrate-binding N domain.

2nd Revision - authors' response 20 April 2010

Referee #1 (Remarks to the Author):

The authors have adequately addressed my concerns.

In their response to the referees, the authors have extensively explained that they were not able to crystallize the WTp97 in the presence ATPgS. They have mentioned in the results section that WT was Monly able to crystallize with ADP and in the discussion that this conformation was resisting crystallization. They have made clear in the publication that according to their SAXS data, the wild type ND1 domain is likely to undergo similar conformational change, but that the IBMFD mutations alter the affinities of D1 for ADP.

On a minor issue, as p97 full-length and N-D1 domains have previously been solved bound with ADP, it would be nice if the authors were to mention if any differences is observed between their structure and the previously published ones.

Overall, I am in favor of publishing the manuscript.

> We thank the reviewer for his/her support of publication of our manuscript in the EMBO Journal.

The reviewer made a request as a minor point, wanting to know the difference between our structure and those of previously determined by others. Although this request is not very clear, we guess that the reviewer is asking about the difference between the ADP bound structure we determined for the mutant R155H and those previously determined wild type p97 with bound ADP, because the differences between the ATPγS- and ADPbound structures are the focus of discussion of this manuscript. I should point out that the differences between mutant and wild type p97 in the presence of ADP were also discussed in our manuscript in different contexts. Structurally, we observed that the Ndomains are in the Down-conformation, but we did not observe any difference within the nucleotide-binding pocket that can be confidently interpreted as significant due to the limited X-ray diffraction resolution (3.4 Å) of our crystals; we also reported the rms deviation between our mutant structure and the wild type structure in the presence of ADP (Page 10 and Table S1). Biochemically, IBMPFD mutants display significant changes toward the binding of ADP both in affinity and in stoichiometry when compared to the wild type enzyme as demonstrated by ITC experiments (Page 16 and Table 4). Clearly, the biochemically observed difference in ADP binding for mutants and wild type p97 is not resolved by crystallographic studies possibly due to limited diffraction resolution. In the Discussion section of the manuscript (Page 22-23), we discussed these observations or differences in the context of our proposed ADP-locked and ADP-open states. Based on the existence of already extensive description and discussion on comparisons between wild type and mutant p97 in the presence of ADP, we feel that any additional description or discussion would only result in redundancy rather than clarity to the manuscript.

Referee #3 (Remarks to the Author):
The authors have addressed the points made in my original review. The paper is significantly clearer and the authors have made appropriate references to the role of D2 in the full-length enzyme. The arguments for the different conformations being nucleotide state dependent are solid. The outstanding question is the relevance of the observed changes to what is happening in the full-length enzyme. Much more work will be needed to understand how the principal ATPase activity provided by D2 relates to the changes seen here. Although how changes are driven remain unclear, I believe that the paper represents a significant advance in understanding the coupling of conformational change to nucleotide state in this class of AAA proteins by defining changes in the N-D1 linker region and its effects on the position of the substrate-binding N domain.

>We thank the reviewer for his/her recommendation in support of publication of our work and we concur with the reviewer that more works are needed to understand the mechanism of communication between D1 and D2 domains.

