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Direct Observation of Stepped Proteolipid Ring Rotation in *E. coli* F_oF_1-ATP Synthase

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1st Editorial Decision 26 May 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. Let me first of all apologise for the delay in getting back to you with a decision. Unfortunately, two of the referees were not able to return their reports as quickly as initially expected.

Your manuscript has now finally been seen by three referees whose comments to the authors are shown below. As you will see while all three referees consider the study as interesting in principle, referees 2 and 3 think that the molecular nature of the "tether" needs to be analysed in more depth and that the study has not yet reached the level of deeper understanding that would be required to be of interest to a broader readership. Given the interest expressed by the referees in principle I have come to the conclusion that we would be able to consider a revised version of this manuscript. However, the molecular identity of the "tether" needs to be characterised further and the other points put forward by the referees also need to be addressed or responded to in an adequate manner. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript as well as on the final assessment by the referees.

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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

Addressing the stepped rotary motion of the c-ring of the ion motor of FOF1-ATPase the authors considerably increased the time resolution and S/N-ratio compared to related work by other labs (PG + MB). This led to the identification of transient dwells followed by the expected 36° jumps which they attributed to the electrostatic interaction of the Arg+ on subunit a with the Asp-
on the adjacent copy of subunit c on the c10-ring. At the high data quality this is novel and merrits publication after some minor revisions (see below).

Title and throughout:
Tether should be renamed, the authors may consider "transient dwell".

p2,II Whether or not the observed dwell time limits the maximum turnover of proton translocation, as stated by the authors, is debatable because in the cited work on proton turnover (refs. 11,28) there has been no indication for rate saturation at high driving force. A conservative statement is that the dwell time is not in conflict with so far reported turnover rates.

Referee #2 (Remarks to the Author):

Report for Authors and Editor

In the manuscript "Direct observation of Fo-dependent rotational tether formation in the FoF1-ATP synthase" the authors Ishmukhametov, Hornung, Spetzler and Frasch used nanodisc-reconstituted F1Fo-ATP synthase from E. coli (n-F1Fo) with a gold nanorod attached (on the Fo-rotor ring) to measure 36° stepped rotation of the Fo motor (result 1) and (reversible) events in each of the substeps, which they named with tether formation (result 2).

One of the models for the functionality of the Fo motor has been described in the literature as a Brownian ratchet; the results in this manuscript support this view twofold by result 1 and 2. Furthermore, the tethering is shown to be independent of the rotor-stator residues cD61-aR210, which are well known to be crucial for ion translocation.

Whereas the 36° stepping of the E. coli Fo motor (result 1) is not novel and confirms previous work (Duser et al., EMBO J. 28, 2009), the tethering aspect of the individual steps (result 2) in a ratchet-type mechanism is new and could not be experimentally addressed until now.

The experimental approach by which rotation is measured is technically very advanced. I am not aware of any other work, which shows such highly resolved rotation experiments in the microsecond timescale and the advanced techniques that are used in the Frasch lab are impressive. Furthermore, the aspect with nanodisc-reconstituted ATP synthase (n-F1Fo, heterologous expression systems allowing mutations/cys-less constructs) seems an attractive new way how to stabilize and conserve enzyme assembly and enzyme activity, respectively. This has potential for broader applications on other membrane protein complexes. The manuscript combines single particle biomechanics with biochemistry. From this point of view the manuscript merits attention and has potential for a broad readership.

The novelty of this manuscript with respect to results and advances in understanding the ATP synthase as a biological machine is restricted to the formulation of the observed tethering effect in a ratchet-type of motor mechanism. The experimental evidence seems clear enough, albeit maybe difficult and not always straightforward to understand for a broader readership. However, my biggest restriction towards this manuscript is that the nature of the tethering itself finally seems to remain obscure and therefore, broader implications also for other ion driven molecular (rotary) motors are difficult to derive at current stage.

The manuscript would profit very much if the authors could strengthen their statement by for example pinpointing molecular interactions (between rotor-stator), which are responsible for the tethering. In Figure 5B and 6, the authors show a tether (red bar) established between subunit a and the c-subunit ring on the cytoplasmic side of the Fo motor. Do the authors imply here something specific, which would be worth discussing? I am completely aware of how advanced the method for measuring the tethering is but for example mutants with no tethering effect would be extremely valuable and highly appreciated; such a result would significantly increase the general relevance of this finding, would be crucial for a deeper understanding the mechanism of the Fo motor and finally make it more interesting for people outside the biophysical field.

For the discussion section I have some questions: I am wondering what is actually the advantage for the Fo motor to have rotational diffusion restricted to 36°, could this maybe help avoiding ion leakage? The possible role of the tethering could be mentioned in the last paragraph of the discussion (or already in the Introduction, second paragraph, last two sentences). What about motor efficiency: does the motor become more efficient if the 36° step is tethered compared with a non-tethered motor? Or is tethering rather meant as a control element for directionality? I am asking this because an unloaded (charged) binding site on the rotor ring obviously faces high energetic restrictions upon exiting the rotor stator interface into the hydrophobic membrane environment (ATP synthesis direction). Could this energetic restriction have anything to do with the observed tethering too? If yes, this aspect would deserve to be mentioned in the discussion too.

The reviewer furthermore suggests looking into the following points, some of them of minor importance:

Abstract:
What is a flashing ratchet? I know the term "Brownian ratchet" and it is certainly known in the field of Biophysics but "flashing" maybe not.

Introduction:
- Third line: it would be good to add references for the ab2c10 stoichiometry of the E. coli Fo complex
- Reference 25 comes after reference 5, then it continues with Ref 6 again.
- Does Reference 16 ("ATP synthesis without aR210") contradict the statement made in the second paragraph at line 6 and 7 saying that aR210 is required to deprotonate cD61?
- Third paragraph, line 7: ....that occurred to(o?) quickly....
- I can´t follow the argumentation in the third paragraph, last sentence ("Since a proton is translocated..."): If one ATP is synthesized every 37 ms (Ref. 24), ten substeps for three ATP would give: 3x37 ms (for three ATPs) / 10 substeps (in Fo) = 11.1 ms for each 36° step. Why can this not be resolved given the 2 ms time resolution of FRET?

Results:
- The authors write that the Fo needed to be stabilized and therefore they have inserted it into nanodiscs. Since this has some interesting novelty and indeed promises to stabilize the enzyme activity significantly, the reader could be interested in more details here: What caused "instability" in Fo and does the incorporation of the Fo into a nanodisc somehow influence the rotation (speed/efficiency) of the rotor (c-ring)? Maybe one would appreciate some controls here to show that this was not the case? This could be an important control to completely rule out that the nanodisc itself has any influence on the rotation measurements (and the stepping effect).
- Page 5, third paragraph, Reference 7: The authors should maybe mention what a "Newtonian fluid" is or does.

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Methods:
- There are many variations and methods to do 2D (polyacrylamide?) gel electrophoresis. The authors should maybe describe the exact method (or give a reference) either in the legend of Figure 1 or in the methods section.
- How was the biotinylation done? How was the attachement on the glass surface performed? Please mention more details here or give reference(s)
- Maybe the authors could also give Reference 26 in the Methods for the abbreviation MSP-1E3D1 (page 9 first line)
- Page 9, last line of first paragraph: How was the formation of 60-70% of n-F1Fo judged/observed?

The following control experiments deserve to be shown:
- DCCD inhibitions/coupling of n-F1Fo compared with F1Fo
- Page 5, third paragraph, line 5: could the authors show this mutant (a-delta14) on the gel? Is there s shift of the a-subunit visible? Since this a-subunit mutant is quite "unusual" (how did the authors actually find this?), the reviewer has some doubts about correct enzyme assembly here.

Discussion:
There is some repetitive element in the second and third paragraph (page 7): "...proton tranlocation is independent of, and not rate-limiting to, the substep interactions..."

Figures and Figure legends:
- there are capital letters for the figures (A, B, C, ...) and small letters (a, b, c, ...) used elsewhere.
- Figure 1A (and results): whereas the activity (and DCCD inhibition experiments) seem to show an (astonishingly well) coupled enzyme, the alpha, beta and gamma bands are considerably strong with respect to the rest of the subunits. The authors claim that the enzyme is correctly assembled. This is somewhat puzzling because in Ref. 31 the distribution is more balanced and typical for F1Fo. Does the preparation contain a (sub)portion of a3b3gamma complexes? Because of this distribution it would be worth to see the full length of the native gel lanes shown in Figure 1A. Maybe some of the subunits get lost somewhere on the way? This point, however, is of minor importance because Figure 1C and D show only blinking when the enzyme is fully assembled.
- Figure 1B: the authors could describe how the F1Fo model was created. Did they use structure files from the pdb database and if so which ones? The figure does not give the impression that the enzyme was bound via 6xHis tags of the beta subunits to the glass surface. It rather implies that the connection is established somewhere on the green subunit (OSCP)? The composition of the outer stalk represents an ATP synthase from eukaryotic sources (bovine?) but certainly does not show the subunits which are present in an E. coli ATP synthase (b2 and delta). I would suggest here in the case the authors use structures in their model, such details should be considered. If the authors just would like to show the overall schematics, then I recommend using a cartoon model (see also Figure 4A)

Figure 2:
How was F1 (used for the experiment shown in Fig. 2A) prepared? Give more details (maybe in Materials and Methods section.

Figure 3C: the substeps and duration could be somehow indicated.

References:
The formatting of the references should be carefully checked. There are many little things which can be improved. Some examples: Species names in italic, F1F0 subscript (of 1 and 0), H(+) superscript (of +), FEBS, EMBO (instead of Febs, Embo)

Referee #3 (Remarks to the Author):

The manuscript by Ishmukhametov and colleagues reports single molecule observation of E. coli ATP synthase as detected by gold nanorods attached to the proteolipid ring of lipid nanodisk reconstituted enzyme. Depending on viscous drag (adjusted via PEG400), some of the observed

molecules undergo 36º sub-steps in the ATP hydrolysis driven rotation of the proteolipid ring, a finding not all too surprising based on ref. 24. However, the current study goes beyond reporting a 36º sub-step in several ways. (1) the here reported time resolution (5X10-6 s) is significantly higher than in the previous study (~10-3 s; ref. 24), allowing an overall more thorough analysis. (2) the authors mention that the presence of sub-steps is not depending on actual proton transport based on an analysis of mutants Asp61G and Arg210G (no data shown). (3) A mutant a-insert-14 was identified that did not show sub-steps under the conditions tested. From these experiments, the authors conclude that the sub-steps must be stabilized by a 'tether' of some sort that limits rotational freedom to 36º steps. However, tether formation can only be observed below a threshold rotation speed, established here by varying PEG400 concentrations. The authors speculate that the function of the tether may be to prevent wasteful ATP hydrolysis in vivo. Overall, the manuscript offers some intriguing new information and possible explanations, but at the same time, some of the results and analyses may be a bit preliminary in nature.

Specific points:

(1) Generally, more detail needs to be provided in terms of statistics (how many sub-steps were measured for each condition/mutant, what's the range of measured time constants for the sub-steps etc.) and experimental methods (ATP, Mg2+, DCCD concentrations, buffers, calculation of drag in Fig. 4A etc. Data analysis should be described briefly in addition to referring to previous publications. Some of this information could go into a supplement.).
(2) Fig. 2A: Is the measurement limited to 90º or is there another reason the authors only show this angular range? One or several full 360º rotations should be shown to see how the ten 36º sub-steps fall into the three 120º (40+80) steps of the F1.
(3) A rate of 6135 H+/sec (163 μs sub-step) corresponds to a F1 turnover of 1530 ATP hydrolyzed per second (assuming 4 H+/ATP; ref. 30 and subsequent work by Turina et al.). The bulk turnover (page 4) is only 140 1/s. How do the authors explain this discrepancy?
(4) Example traces for c-Asp61G and/or a-Arg210 that show sub-steps should be added to Fig. 2A.
(5) How was mutant a-insert-14 identified? Does a-insert-14 with an intact Arg210 function properly in that ATPase is coupled to proton translocation and E. coli can grow on e.g. succinate?
(6) If a-insert-14 with Arg210 translocates protons but shows no sub-steps, this mutant would provide a way to test the author's hypothesis of the significance of the sub-steps in vivo.
(7) Fig. 2A and 3B: It appears that the three steps shown (for 15% PEG) add up to ~90º instead of 108º. What's the overall accuracy/error of the angle determination based on gold nanorod observation? See point (1) above.
(8) Each powerstroke is 120º but each sub-step is 36º. Can the authors offer an explanation as to how the mismatch could be overcome? See also point (2).
(9) What was the ATP concentration? Did the authors try limiting ATP concentrations to slow down the enzyme instead of increasing PEG400?
(10) Did the authors observe any backward sub-steps?
(11) How do the authors explain their observation (page 6): "Any molecule that rotates 36° in <163μs will not exhibit sub-steps"? Is the rotation rate at <163 μs too fast to form or too fast to observe?
(12) Did the authors observe double, triple or multiple sub-steps as in ref. 24?
(13) The authors use sub-step duration for the time the gold nanorod is stationary. Shouldn't this time better be called 'dwell-time'?
(14) Can the authors speculate as to the nature of the 'tether' between subunit a and c?
(15) According to the Nyquist-Shannon sampling theorem, shouldn't an acquisition speed of 100 (200) kHz result in a maximum resolution of 20 (10) μs instead of 10 (5) μs?
(16) The title of the manuscript needs revision. It's not clear what the authors mean by "(Fo-Dependent) Rotational Tether". Maybe change to something like "Direct Observation of Stepped (Proteolipid Ring) Rotation in E. coli F1Fo-ATP synthase - Evidence for Transient Tether Formation Between Subunits a and c".

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Additional correspondence 15 June 2010

I want to thank you for the fair and thorough reviews of our manuscript EMBOJ-2010-74557. I plan to respond to all of the points in detail.
However, before I do, I need some clarification from you concerning your decision letter. Specifically, it is your statement, "However, the molecular identity of the "tether" needs to be characterized further". It is important that I get it right due to the single revision round policy.

The specific comments of Reviewers 2 and 3 concerning this point are as follows:
Ref 1. "The manuscript would profit very much if the authors could strengthen their statement by for example pinpointing molecular interactions (between rotor-stator), which are responsible for the tethering. In Figure 5B and 6, the authors show a tether (red bar) established between subunit a and the c-subunit ring on the cytoplasmic side of the Fo motor. Do the authors imply here something specific, which would be worth discussing? I am completely aware of how advanced the method for measuring the tethering is but for example mutants with no tethering effect would be extremely valuable and highly appreciated; such a result would significantly increase the general relevance of this finding, would be crucial for a deeper understanding the mechanism of the Fo motor and finally make it more interesting for people outside the biophysical field."

Ref 2. "(14) Can the authors speculate as to the nature of the 'tether' between subunit a and c?"

Referee 1 uses the phrase that "the manuscript would profit very much if the authors could strengthen their statement..." Referee 2 only requests that we speculate about the molecular identity of the tether. It is going to take a couple of years of work for us to specifically pinpoint the residues involved in the tether. It is likely that we will make several mutations that will have no effect on the process. This will be well beyond the August deadline for a response to this manuscript. Tassilo Hornung, who appears as a middle author on the manuscript, has made some progress on this topic. It is a great deal of work for which we only have a partial answer at this time. Tassilo deserves to be first author on the paper that identifies these residues.

We will abide by your decision concerning this point, but we just want you to clarify how much new data, if any, you feel that we must present regarding the identity of the tether for this manuscript to be acceptable for publication.

Additional correspondence 17 June 2010

Thank you for your message regarding the conditions for your revision. I have now had a chance to review your arguments and to look into the matter once more. The issue with this additional level of analysis is not that it is needed to make the study sufficiently conclusive (thus publishable in principle). The issue is rather which level of deeper understanding is needed before the study is of sufficient significance to appeal to a broader readership and thus to reach the bar for publication in The EMBO Journal. Referee 2 in fact feels rather strongly that such deeper insight is required to reach this bar and in fact indicates in his/her overall rating returned to the office that the study may be better suited to publication in a more specialised journal in its present form.

Now I can see that it is not trivial to gain such deeper insight and to nail down the exact residues that are involved in the rotor/stator interaction. Still, it would be important to take the study further in this direction and to at least provide a deeper mapping of the domains/regions required. We will certainly be prepared to extend the deadline for your revision if this is required.

I hope that these thoughts are helpful. I am looking forward to your revision in due course.

Yours sincerely,

Editor
The EMBO Journal

1st Revision - authors' response 13 August 2010

Referee #1

1-1 Tether should be renamed, perhaps "Transient Dwells"
We adopted Transient Dwells as requested (see also Reviewer#3) and we substituted the word leash for tether.

1-2 Whether or not the observed dwell time limits the max turnover of proton translocation is debatable…
We agree that this is an important point and have now included new statements as suggested by the reviewer to address this issue on p.3, ¶3; on p.10, ¶3, and on p. 11, ¶4.

Referee#2

2-1 The authors could strengthen their statement by for example pinpointing molecular interactions responsible for the tether.
Figure 5 now addresses this issue to explain in more detail how the results provide evidence of the location of the molecular interactions. We also now provide a more detailed explanation of the results as they impact this question on p.7, ¶2l; and on p.10, ¶1. Based on the subunit-a information provided in Figure 5 and these crystal structures, it is very unlikely that the source of the "leash" (formally tether) occurs anywhere else except for subunit-a helices 3C and 4C, with the cytoplasmic side of the c-ring.

2-2 *Add to the Introduction and Discussion the potential advantage(s) of the 36° restricted rotational diffusion.*
We complied by adding the last sentence to the Introduction and the last paragraph in the Discussion.

2-3 What is a flashing ratchet?
We complied by removing flashing.

2-4 Add references for ab2c10.
We complied.

2-5 Correct numbering for reference 25.
We complied.

2-6 Does Reference 16 ("ATP synthesis without aR210") contradict statement that aR210 is required to deprotonate cD61?
This is now reference-19. No, this is not a contradiction, but the title of that reference is misleading. In reference 19, data is shown for double mutant aR210Q/aQ252R that swaps the Q and R to the equivalent positions on adjacent transmembrane helices. Thus the R is still required.

2-7 "occurred to(o) quickly".
We have now removed that sentence.

2-8 Can't follow argument, "Since a proton is translocated…" Why can this not be resolved given the 2 ms time resolution of FRET?

We have now rewritten for clarity where we agree with the reviewer that it is possible that this can be resolved if it occurs slowly enough. We modified p.3, ¶5 where this statement originally appeared, and we added Figure 10 and the associated Discussion on p.10-11 to explain this issue in greater depth.

2-9 (a) What caused the "instability" in Fo and
(b) does the incorporation of the Fo into a nanodisc somehow influence the rotation (speed/efficiency) of the rotor (c-ring)?
(c) Show controls that this was not the case? Important to rule out that the nanodisc itself has any influence on the rotation measurements (and the stepping effect).
The basis for our conclusion that the nanodisc itself does not negatively influence the rotation measurements is summarized on p.7, ¶4 last 3 sentences. We have now included Figure 1C that shows how ATPase activity of the enzyme is stabilized by the nanodiscs relative to detergent solubilized enzyme, which is described on p.4, ¶3. Page4, ¶4 now includes a description of the comparison of the abundance of molecules observed to rotate when incorporated into nanodiscs relative to detergent solubilize enzyme. We also added text on p.7, ¶3 and 4 that shows that nanodiscs to not negatively impact the torque generated.

2-10 Explain what a "Newtonian fluid" is or does.
We have complied. The explanation is included in the additional text on p.7, ¶3.

2-11 The authors should describe exact methods used for 2D electrophoresis or give a reference.
We complied in the Methods.

2-12 Biotinylation methods, and attachment to glass surface methods. Provide details or give reference.
We complied in the Methods.

2-13 Authors could also give reference 26 in Methods
We have included this reference (now ref 29) in the Methods.

2-14 How was the formation of 60-70% n-FoF1 judged/observed.
We have now provided that information on p13, ¶4.

2-15 Show DCCD inhibitions/coupling of n-FoF1 compared to F1Fo.
We have complied in Table I. We now include text on p.4, ¶3 describing these results and explaining that this extent of inhibition is typical for DCCD modification as reported by Ueno (ref 26) and elsewhere.

2-16 Show the PAGE of the a-delta14 mutant. Is a shift in subunit-a visible? Reviewer doubts correct enzyme assembly of mutant.
We have complied in Figure 5B. There may be a small shift in the subunit-a band. The protein has clearly assembled.

2-17 There is repetition in the 2nd and 3rd paragraphs "proton translocation is independent of, and not rate limiting to, the substep interactions…"
We have rewritten this section as requested.

2-18 Capital letters used in Figures and small letters used elsewhere.
We corrected these inconsistencies.

2-19 Figure 1A: whereas the activity and DCCD experiments seem to show an astonishing well coupled enzyme, the ạḅg bands are considerably strong with respect to the other subunits. Does the preparation contain a (sub)portion of α3b3γcomplexes? It would be worthwhile to see the entire native gel lanes.
We have complied by showing Figure 1A.
Reviewer #2 noted that this comment is of minor importance and notes that we have already shown that Figures 2B and C (formally Figures 1C and D) answer his question. The 85% inhibition by DCCD that we report is not unusual but merely average as we now state on p.4, ¶3 quoting Ueno et al. (ref 26). In addition, the denaturing gel that separated the subunits (Figure 1B) was stained with

silver. Although silver stain is the most sensitive means to see all contaminants, it does not stain bands in proportion to their relative abundance in a protein complex.

2-20 Figure 1B: the authors could describe how the F1Fo model was created…I recommend using a cartoon model (see also Fig. 4A).
We have complied and now show cartoon models.

How was F1 prepared? Give more details in Methods.
We have complied.

2-21 Figure 3C: the substeps and duration could somehow be indicated.
We have complied by adding arrows to what is now Figure 4.

2-22 Check formatting of references.
We have complied.

Referee #3

3-1 Provide more detail in terms of statistics:
(a) how many substeps measured for each condition/mutant;
(b) what's the range of measured time constants for the substeps etc;
(c) data analysis should be described in addition to referring to previous publications.
Some of this can go into a supplement.
We have complied by adding this information to Table II and we added error bars wherever possible. We added Figure 3 to explain the data analysis in more detail and we added the section "High Speed Rotational Power Stroke Measurement using Gold Nanorods" on p.5-6. We also added detail concerning the data analysis in the Methods on p.14 under the section "Single Molecule Studies". We explain now in further detail that we typically obtain about 3520 transitions in a 50 second data set from each molecule.

3-2 (a) Is the measurement limited to 90 degrees or is there another reason that only this angular range is shown?
(b) One or several full 360 degree rotations should be shown to see how the ten 36 degree substeps fall into the three 120 degree steps of the F1.
We do observe continuous rotation for several thousand cycles. However, our quantitative analyses are based on 90 degree intervals because those are most accurately measured by our approach. We have now explained how and why we do the analysis in Figure 3 and associated text in Results as specified above on pp. 5-6.

3-3 A rate of 6135 H^+/sec (163 μs per substep) corresponds to a F_1 turnover of 1530 ATP hydrolyzed per second assuming $4H^+$/ATP ref 30 and subsequent work by Turina et al.). The bulk turnover (page 4) is only 140 per sec. How is this discrepancy explained?

The rate of 6135 H^+/sec was obtained with F_o in the absence of F_1. Under these conditions proton translocation can go extremely fast- in fact, the rate does not saturate at even the highest proton gradients as pointed out by Reviewer #1 (see 1-2). The rates reported in ref 30 (now ref-43) and by Turina et al. were done with F_oF_1 and are therefore slower. Thus, published reports that the H^+ transport rate can go much faster and does not saturate do not present a discrepancy, rather they provide independent confirmation to the results presented here that proton transport (including aR210 and cD61) is not the rate-limiting part of the mechanism.

The difference in the 6135 H^+/sec and the ATPase rate is due to the fact that the F_1 pauses for ~8.3 ms during the catalytic dwell. The rate of rotation during the power strokes between F_1-dependent catalytic dwells, when protons are transferred, occurs at a much faster rate. We have now added Figure 10 and the text on pp.10-11 to explain this relationship.

3-4 Example traces for cD61G and/or aR210G that show substeps should be added to Figure 2A.
We have complied in Figure 4B.

3-5 (a) How was the mutant a-insert-14 identified?

(b) Does a-insert-14 with an intact R210 function properly? If a-insert-14 with R210 translocates protons but shows no substeps, this mutant would provide a way to test the authors' hypothesis.
We now include a statement on p.6, ¶5, "The subunit-a mutation designated n-F_oF_1-a∇14 was formed by site-directed mutagenesis during PCR at suboptimal conditions, and identified by sequencing." The inserted sequence duplicates the hydrophobic region of subunit-a TMH-3 such that aR210V as well as "aR210G" are present. If only one copy of an aR210 mutant were present, we would agree that it would be worthwhile to determine the effects of restoring the arginine. However, it is less certain that a clear answer would be achieved by analysis of the enzyme in which one or both aR210 residues had been replaced. Figure 5 now addresses this issue to explain in more detail how the results provide evidence of the location of the molecular interactions. We also now provide a more detailed explanation of the results as they impact this question on p.7, ¶2l; and on p.10, ¶1.

3-6 Figures 2A and 3B: It appears that the 3 steps shown for 15% PEG add up to 90 degrees, instead of 108 degrees. What is the accuracy/error of the angle determination based on gold Nanorod observation? See point 3-1.

The 3 steps shown are example transitions. The average stepping observed from 1000's of transitions is ~37 degrees as is now reported in Table 2. We have added Figure 3B and C to provide the information requested that includes the standard errors on the measurements. We also added text to explain how the data were obtained. In addition we added Figure 10 and text to the discussion to address this issue, particularly in the context of the results presented in ref-27.

3-7 Each power stroke is 120 degrees but each substep is 36 degrees. Can the authors explain how this mismatch could be overcome? See also point 3-2.
We added Figure 10 and associated text (p.10, the 2nd and 3rd full ¶s) to address this question.

3-8 (a) What was the ATP concentration?
(b) Did the authors try limiting ATP to slow down the enzyme instead of increasing PEG400?
We now specify in the text that we used 1 mM $MgCl_2$ and 2 mM ATP for a working concentration of 1mM Mg-ATP on p.5, last ¶, and p.14, ¶3. This is a saturating concentration such that rotation occurs in 120 degree steps separated only by the F_1-dependent catalytic dwells. We did not try limiting ATP concentrations because this does not slow down the power strokes of the enzyme. Instead this would cause the appearance of the F_1-dependent ATP-waiting dwell 30-40 degrees from the catalytic dwells, and would further complicate the observation of the F_o-dependent transient dwells.

3-9 Did the authors observe any backward substeps?
As is apparent in Figure 4 (formally Figure 2A and B) and Figure 7C that some of the transient dwells appear to have a "recoil" effect in which backward rotation by a few degrees is observed. We now mention this effect on p.6, ¶3.

3-10 How do the authors explain their observation (p.6): "Any molecule that rotates 36 degrees in <163 μs will not exhibit substeps." Is the rotation rate to fast to form or too fast to observe?
The duration of the transient dwells becomes longer as the rotation rate increases up to the 163 ms limit (for 36 degrees). Given this trend, the transient dwell should get longer, not suddenly become so short that we are unable to observe it. The result that the transient dwells do not form at high rates of proton transport is consistent with the points previously raised (point 3-3 and reviewer #1-2). This point was also addressed in response to Reviewer#2-2, who asked us to add text to describe the potential advantages of the transient dwells.

3-11 Did the authors observe double, triple or multiple substeps as in ref 24?
Multiple transient dwells did not occur to any significant extent, and certainly not in the abundance of ~50% reported in ref-27 (formally ref-24). This is now addressed in the text added on p.6, ¶3 to describe Table II, and in the discussion regarding Figure 10 on p.10, the 2nd and 3rd full ¶s.

3-13 The authors use substep duration- shouldn't this time better be called 'dwell time'?
We have complied by referring to this now as the "transient dwell" time as suggested by all of the reviewers. We had chosen the original moniker to distinguish it from the F_1-dependent "catalytic" and "ATP-waiting" dwells.

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3-14 Can the authors speculate as to the nature of the 'tether'?
Figure 5 now addresses this issue to explain in more detail how the results provide evidence of the location of the molecular interactions. We also now provide a more detailed explanation of the results as they impact this question on p.7, ¶2; and on p.10, ¶1. Based on the subunit-a information provided in Figure 5 and these crystal structures, it is very unlikely that the source of the "leash" (formally tether) occurs anywhere else except for subunit-a helices 3C and 4C, with the cytoplasmic side of the c-ring.

3-15 Report maximum resolution as per Nyquist-Shannon sampling theorem.
We have complied.

3-16 Change the manuscript title.
We have complied.

2nd Editorial Decision 07 September 2010

Thank you for sending us your revised manuscript. Our original referees have now seen it again. While they are now more positive regarding publication of the study here referee 2 is still hesitant and expresses this in his/her overall rating returned to the office and referee 3 still thinks that the nature of the leash (tether) needs further clarification. Taking together these two votes I think that the case is borderline. I have therefore come to the conclusion that it would still be important to address referee 3's concerns, and I would like to ask you to do so in an amended/re-revised version of the manuscript and to also address the remaining points raised by referee 2.

I am looking forward to receiving your amended manuscript.

Yours sincerely,

Editor
The EMBO Journal

Referee #1 (Remarks to the Author):

The revision has improved the manuscript, which appears ready for publication.

Referee #2 (Remarks to the Author):

The reviewer is pleased to see that the manuscript of Ishmukamteov et al. has been worked out more precisely in many respects and was improved. New figures (now a total of 11!) and two tables have found place and the text itself has been extended. The resolution of the rotation experiments is unprecedented indeed (as stated in the Abstract) and impressive.
The explanations have become more precise; the text in all subsections is more detailed, maybe sometimes even a bit lengthy. The Brownian ratchet is explained more clearly. The reader can understand well why the observed transient dwell is an important feature of the ratchet mechanism as it fulfils the "second requirement of the ratchet to restrict the rotational diffusion of the c-ring" as written in the manuscript Discussion section.
The biochemical assessment of the nature of the nanodisc-incorporated ATP synthase has been presented with more experiments (e.g. Figure 1C and Table 1). The work profits from these improvements. (The suggested reference formatting, however, was not yet improved.)

The question of the possible molecular interaction between the a-subunit and the c-ring with respect to the investigated tether effect (now described as "leash") has been assessed in the manuscript in the framework of the experiments already presented in the first manuscript version and the results obtained. The authors indicate that charged residues on the c-ring (cD44 and cR50 in E. coli) form a

periodic interaction by salt bridge(s) with the a-subunit helices 3C and 4C. Can this statement maybe be supported by the crosslink data that has been observed by the Fillingame lab? And do secondary structure predicted models of the a-subunit go conform to these statements as I believe that cD44 and cR50 lies already in the water soluble (cytoplasmic) part of the c-ring? This is maybe something to consider more precisely on page 7, second paragraph. As no new experimental results are presented in the revised manuscript, which could validate the authors´ statements, the exact molecular nature of the leash can maybe be clarified in future work. However, the revised work is a valuable technical advance and contribution to the ATP synthase motor mechanism.

Referee #3 (Remarks to the Author):

In the revision of their manuscript, the authors have addressed most of the points raised in the first round of review in a satisfactory manner and as a consequence, the manuscript has improved significantly. However, a few issues related to the nature of the subunit a-insert14 mutation and the putative subunit a-c leash remain that need further clarification:

- From the way the authors describe how they generated mutant a-insert14 it almost sounds as if the mutation was not intended ("during PCR at sub-optimal conditions", page 6). Since the existence and nature of the putative 'leash' between subunit a and c is a (if not the) major novel finding in the study, and since the a-insert14 mutation represents the only experimental evidence that the leash can be disrupted, generation of the mutation should be fully explained: was the mutation intended? ... and if not, which mutation was? (aArg210Gly?). Did the authors look for mutations that disrupt the leash? etc.

While the author's explanation for the origin of the leash is plausible, they could have nailed it with changing (some of) the putative charged residues in the cytoplasmic loops of subunit a and c and look for presence or absence of the transient dwell. As it stands, the reader is left with some uncertainty as to the effect of the a-insert14 mutation on enzyme functioning and assembly (despite the author's confidence in response to reviewer 2's point 16 that the enzyme "has clearly assembled". The added SDS gel of a-insert14 shows a band above and a band below the position of the a subunit band in the wild type lane. It is also questionable whether a SDS gel that appears to show all subunits can count as proof that the enzyme is correctly (functionally) assembled).

- It is likely that the inserted 14 residues extend TMH 4 of subunit a on the cytoplasmic side (as drawn by the authors in Fig. 5C), and therefore replacing V210 in the original sequence by Arg and testing for enzyme function could provide support for the author's model (as drawn in Fig. 5; The author's response to this reviewer's point 5b wasn't so clear).

- Page 10: Briefly describe the effects of mutations aE196 and K203 as published earlier by others and why these mutations are more consistent with the effects of a-insert14 described in the current manuscript.

- Page 10: The 1st sentence of the 2nd paragraph is not clear. What mechanism?

- Fig. 9 and 11: Tether needs to be replaced by leash.

2nd Revision - authors' response 17 September 2010

Please note that we moved the detailed description of the F1 preparation (requested in revision 1) to supplementary material in order to accommodate the additional text requested during this revision and remain within the length requirements of EMBO J.

Reviewer #1: Accept for Publication.

Reviewer #2:

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1- The authors indicate that charged residues on the c-ring (cD44 and cR50 in E. coli) form a periodic interaction by salt bridge(s) with the a-subunit helices 3C and 4C. Can this statement maybe be supported by the crosslink data that has been observed by the Fillingame lab? And do secondary structure predicted models of the a-subunit go conform to these statements as I believe that cD44 and cR50 lies already in the water soluble (cytoplasmic) part of the c-ring? This is maybe something to consider more precisely on page 7, second paragraph. As no new experimental results are presented in the revised manuscript, which could validate the authors' statements, the exact molecular nature of the leash can maybe be clarified in future work.

The second and third paragraphs of the Discussion (page 10) now include a more detailed and referenced description of the data that support the structure subunit-a including the evidence concerning which residues face the c-ring, and their location relative to the membrane surface. In addition, the evidence concerning the position of cD44 and cR50 relative to the membrane surface is presented. We went beyond the request of reviewer #2 and provided references of the work of Vik's lab in addition to that of Fillingame's lab that independently support these structural assignments. We also added a sentence that more experiments are required to pinpoint the specific interaction responsible. Rather than put this information on page 7 as suggested by Reviewer #2, we decided that it was better suited to go on page 10 because it is more of a discussion topic, and should not interrupt the presentation of results.

(The suggested reference formatting, however, was not yet improved.)
We further revised the reference formatting and we hope that it is now acceptable.

Referee #3:

1- From the way the authors describe how they generated mutant a-insert14 it almost sounds as if the mutation was not intended ("during PCR at sub-optimal conditions", page 6). Since the existence and nature of the putative 'leash' between subunit a and c is a (if not the) major novel finding in the study, and since the a-insert14 mutation represents the only experimental evidence that the leash can be disrupted, generation of the mutation should be fully explained: was the mutation intended? ... and if not, which mutation was? (aArg210Gly?).
We now state on sentence 1, page 12 under "Preparation of F_oF_1" that the a-insert14 mutant was made by accident in the process of making the aR210G mutant, and explain that it resulted by using a non-optimal annealing temperature during PCR. Yes, it is a major novel finding because for the past 25 years, the aR210-cD61 interaction has been the sole interaction between these subunits to explain the F_o mechanism.

2- Did the authors look for mutations that disrupt the leash? etc.
Analysis of each mutation made takes several months of work. Please see the answer to question 4.

3- While the author's explanation for the origin of the leash is plausible, they could have nailed it with changing (some of) the putative charged residues in the cytoplasmic loops of subunit a and c and look for presence or absence of the transient dwell. As it stands, the reader is left with some uncertainty as to the effect of the a-insert14 mutation on enzyme functioning and assembly (despite the author's confidence in response to reviewer 2's point 16 that the enzyme "has clearly assembled". The added SDS gel of a-insert14 shows a band above and a band below the position of the a subunit band in the wild type lane. It is also questionable whether a SDS gel that appears to show all subunits can count as proof that the enzyme is correctly (functionally) assembled).

This is a certainly a valid point. However, if I understand the question correctly, the only definitive way to demonstrate functional assembly is to compare the crystal structure of the wild type and mutant n-FoF1. Since 20 years of attempts to determine the crystal structure of subunits-a and b in FoF1 have thus far not been successful, the best we can do at this point is to show: (i) that all the subunits have become incorporated into the nanodiscs by a gel, (ii) compare the ability of the enzyme to catalyze ATP hydrolysis, (iii) and demonstrate the ability of the drive shaft (including the c-ring) to rotate, which we have done. We have added a sentence that more experiments are necessary to pinpoint the interaction as recommended by reviewer #2.

4- It is likely that the inserted 14 residues extend TMH 4 of subunit a on the cytoplasmic side (as drawn by the authors in Fig. 5C), and therefore replacing V210 in the original sequence by Arg and testing for enzyme function could provide support for the author's model (as drawn in Fig. 5; The author's response to this reviewer's point 5b wasn't so clear).

I am delighted that Reviewers #2 and #3 both feel that the identification of the molecular basis for the leash is an important question to be answered. Hopefully, the reviewers of my pending grant application will be as enthusiastic and provide the funds required to complete this task. Until such funds are available, I am unable to comply with this request of Reviewer #3.

- Page 10: Briefly describe the effects of mutations aE196 and K203 as published earlier by others and why these mutations are more consistent with the effects of a-insert14 described in the current manuscript.

The second and third paragraphs of the Discussion (page 10) now include a more detailed and referenced description of the data that support the structure subunit-a including the evidence concerning which residues face the c-ring, and their location relative to the membrane surface. In addition, the evidence concerning the position of cD44 and cR50 relative to the membrane surface is presented. We went beyond the request of reviewer #2 and provided references of the work of Vik's lab in addition to that of Fillingame's lab that independently support these structural assignments.

- Page 10: The 1st sentence of the 2nd paragraph is not clear. What mechanism?
We amended this sentence for clarity, particularly now that the preceding text has been expanded to comply with the additional information of the request above.

- Fig. 9 and 11: Tether needs to be replaced by leash.
Leash has now replaced tether in these figures.

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