THE EMBO JOURNAL

Manuscript EMBO-2009-70941

# 36° step size of proton-driven *c*-ring rotation in $F_oF_1$-ATP synthase

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**Review timeline:**

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|---|---|
| Submission date: | 26 March 2009 |
| Editorial Decision: | 05 May 2009 |
| Additional Correspondence: | 12 May 2009 |
| Additional Correspondence: | 03 June 2009 |
| Revision received: | 04 June 2009 |
| Editorial Decision: | 26 June 2009 |
| Revision received: | 29 June 2009 |
| Accepted: | 29 June 2009 |

## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 05 May 2009

Thank you for submitting your manuscript to the EMBO Journal. Three referees have evaluated your manuscript and their comments directly for authors are provided below. I am sorry for the slight delay in getting back to you, but it unfortunately has taken a little bit longer than anticipated to receive the full input on your study. I have also discussed your manuscript and the reports further with our executive editor.

As you can see while the referees find the analysis interesting, referee #2 also raises significant technical concerns with analysis and the conclusiveness of the findings reported. The concerns raised are significant and the referee finds that the manuscript is not well suited for publication in the EMBO Journal. As you can see from the other comments and as referee #2 also indicates, the topic and the questions addressed are clearly of interest. If the technical concerns raised by referee #2 can be satisfactory resolved then we would like to consider a revised manuscript. However for us to do so, these concerns really need to be addressed and this is what is unclear at this stage if they can be resolved. Given this uncertainty, it would be good if you could provide us with a preliminary response to referee #2 and how you would address the concerns raised. We would then involve another expert to take a look at the comments of referee #2 and your response. This will allow us to make a quick decision now to see if submitting a revised version makes sense or not. I recognize that this is perhaps an unusual approach, but I think that this is the most practical way to move forward.

Thank you for the opportunity to consider your work for publication.

Yours sincerely,

 

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The manuscript by Dueser et al. reports the (according to the authors) first direct observation of a $36^o$ stepping of the proteolipid ring of E. coli $F_1F_o$-ATP synthase during catalytic turnover in ATP synthesis mode. The authors perform single molecule FRET measurements with reconstituted ATP synthase labeled with EGFP as fluorescence donor and Alexa568 as fluorescence acceptor in a similar fashion as reported earlier by the same group for establishing the direction of subunit rotation during ATP synthesis (Diez et al., 2004). The two models for c-ring rotation considered by the authors at the onset of their experiment were the $36^o$ and the $120^o$ step models. The radius of the c-ring was assumed to be 2.5 nm and the position of the EGFP donor with respect to the c ring was estimated from previous studies (3.2 nm off axis and 4.2 nm above the plane of the acceptor). The expected distance changes between two positions (e.g. 1 - 2) for both models were plotted as a function of the two distances to the donor to yield two elliptic curves rotated by $90^o$. Observed photon bursts were analyzed manually and classified into five FRET efficiencies, corresponding to five distance ranges. Dwell times were estimated to be 9 +/- 1 ms, consistent with the observed ATP synthesis rate but in average about 3-4 times faster than dwell times of the gamma/epsilon rotor (18-51 ms). Inhibitors were used as controls to reduce the number of rotating ATP synthase molecules (DCCD) and/or to slow them down (aurovertin B). All in all, the FRET steps from close to 3,000 ATP synthase molecules were analyzed to produce a FRET transition density plot (shown twice, in Fig. 3F and 4H) and a FRET distance change plot (Fig. 4A). The data show about 48% $36^o$ stepping with remainder being $72^o$, $108^o$ and $144^o$ steps (37%, 12%, 3%, respectively). Furthermore, Monte Carlo simulation of the FRET distance change plots expected for all possible step sizes matched the observed combination best with still a reasonable fit for the $36^o$ step size only (Fig. 4A vs. B).

Overall, this is a nice manuscript with a clear result based on high quality experimentation and data analysis that should be of interest to the general reader of the EMBO J. The study provides novel insight into the mechanism of ATP synthase in that it can be taken as evidence that the gamma/epsilon rotor serves as an elastic coupling that stores the energy of (e.g.) four $36^o$ steps of the c-ring during pmf driven rotation. However, before publication could be considered, a number of issues need to be addressed:

1) Data analysis: 24% of close to 12,000 molecules had both dyes, 1294 bursts were used for FRET transition density plot and 1879 for distance histograms (page 7). A table summarizing these numbers and where they came from would be helpful.
2) Data interpretation: The placement of the arrows in Fig. 2A and B is not entirely clear. Are the arrows supposed to point to different FRET levels (especially in 2B there are more 'levels' than arrows)? Since these 'bursts' were analyzed manually (as mentioned on page 4, bottom paragraph), maybe include horizontal lines corresponding to the 5 distance bins.
3) Page 4, bottom: How do the authors calculate the 36 1/s? A 9 ms dwell results in 111 protons/s and assuming 4 protons per ATP would give a turnover number of 28 (very close to the measured 27 1/s).
4) Page 5, top: The effect of Aurovertin B binding is not clear. The number of rotating ATP synthases "...was reduced by 75% and the dwell times were prolonged more than 2-fold". How many molecules had both dyes, how many did rotate? Does aurovertin B binding inhibit 100% (prevent rotation) or only slow down activity? Did the authors measure ATP synthesis biochemically with and without Aurovertin? The authors say that residual activity is 50% "... in agreement with biochemical measurements" but no data are shown or paper is cited. Same question for DCCD inhibition: On page 10 it says that 60 µM DCCD abolishes ATP synthesis. On page 4 is mentions an inhibition efficiency of 63%.
5) What could be the source of the discrepancy between observed and simulated FRET transition density plot in terms of probabilities? In the observed plot, most distance changes are seen at the

 

long sides of the ellipse (similar to Fig. S5) whereas in the simulated plot (Fig. 4I), most probability is at the tips.
6) Could it be that the 40° substep in gamma/epsilon (e.g. Yasuda et al., 2001) stepping is actually a 36° step of one c subunit (at least in E. coli $F_1F_o$)?
7) Can the authors speculate as to how the coupling can accommodate the requirement of 4 protons/ATP with only 10 c subunits (protons) per revolution?
8) Overall, the manuscript needs careful editing:
- there are no page numbers
- page 4, bottom paragraph: Affecting instead of effecting or better 'effect of aurovertin B on dwell time..'
- page 5,middle: Fix the sentence "These effects of ..."
- page 7, end of top paragraph: "... shown as Fig. F the manuscript)" Something seems to be missing here.
- page 8, middle: "This might be due to limited the..."
- Fig. 3A is not referred to in the text (and the order of the other parts of the figure is mixed up, with F coming first etc.)
- Same for Fig. 4. Not all parts are referred to in the text. This makes it harder to follow the description of the results.

Referee #2 (Remarks to the Author):

This manuscript is about single-molecule spectroscopic work on the rotation of the Fo rotor in $F_1F_o$-ATP synthase. Using an advanced optical technique to monitor a single-molecule fluorescence resonance energy transfer (FRET), the authors aimed to detect the unitary step of the rotation of the Fo rotor. They presented the time trajectories of the FRET signal from the ATP synthase molecules reconstituted in liposome passing through the laser focus of confocal microscopy, and attempted to resolve the FRET signal into discrete levels corresponding to the 36-degree steps that are expected from the structure of the rotor ring of an Fo motor, comprising 10 copies of c subunits. They also provided the simulation data, to support their arguments vis-a-vis the 36-degree steps.

The cogency of the conclusion drawn by the authors primarily depends upon the validity of the first analysis procedure, i.e., the manual identification of discrete FRET levels from the time trajectories of the FRET signal. However, this procedure is surprisingly subjective. The four examples of time trajectories from the FRET signal in Fig. 2 show attempts by the authors to identify discrete states in the FRET signal, as denoted by black arrows. However, the authors did not provide any criteria for determining FRET levels, in the midst of such noisy data. How can one agree with the authors' identification? People could easily mark these arrows at different positions, or hesitate to mark at all. Among such noise and largely fluctuating signals, manual identification should not be employed. The verification of discrete FRET levels by objective means is definitely required. Any conclusions drawn from such subjective analysis is not acceptable; thus, this manuscript is not suitable for publication.

There are other serious issues:

1. The donor-acceptor distance was calculated from FRET, assuming the isotropic rotation (or distribution) of the fluorophore of EGFP. However, the tethering of EGFP to the C-terminal of a subunit would restrict or bias the rotation of EGFP. The authors may claim an apparently low EGFP anisotropy; however, they should note that this does not guarantee the isotropic distribution of the angle of the EGFP.
2. It is not at all clear, how the authors deduced the unidirectional rotation from the given trajectories of the FRET signal, which does not provide information regarding a single turn of the *c*10-ring. A random rotation with a 36-degree step is also able to reproduce the data. Have the authors carried out the requisite control experiment, without a proton motive force? The findings of such an experiment should be provided, to discriminate experimentally the unidirectional and random motions. For this purpose, simulation data is not required.
3. Taking previous studies into account, it is strange that Fo makes steps of 36 degrees each, in 13 ms, in the ATP hydrolysis. Woodbury's group determined the velocity of proton translocation to be 3,100 protons/s (Franklin et al., Biophysical Journal 2004). This means that the waiting time of $F_o$ for each proton translocation is only 0.3ms, too short to be detected. This finding is consistent with

 

the Yoshida group's report (Ueno et al., PNAS 2005), that Fo neither affects nor limits rotation; they observed 120-degree stepping rotation of Fo but not any small substep in the ATP-driven rotation. Thus, there is a serious discrepancy between this study's finding of a 36-degree step in hydrolysis and the findings of previous studies. An explanation to this effect should be provided.
4. Another discrepancy is the torsion energy in the rotor complex, due to the difference in rotational step size between $F_o$ and $F_1$. The authors claim in this work that $F_o$ makes 36-degree steps, while they previously reported that the $F_1$ portion undergoes a 120-degree stepping rotation in the whole complex of ATP synthase, even in ATP synthesis. If this is the case, it means that the rotor part is transiently distorted up to 84 degrees. How does the rotor complex store such large torsion? Junge's group reported the stiffness of the parts of the rotor, which should store the torsion if it happens to be 68 pNnn/radian. This means that the backward torque exerted by the rotor should be -100 pNnm. It is apparently impossible for the $F_o$ motor to overcome such a large energy gap.
5. In Figs. 2E and 2F, the distributions of dwell time are shown. However, the ATP synthesis rate strongly depends on the amplitude of the proton motive force, which dissipates with time. Under the experimental conditions used, for how long was the proton motive force sustained? In previous studies, including two referenced by the authors (i.e., Steigmillar et al., PNAS 2008 and Fischer et al., FEBS Lett 1999), the proton motive force dissipated relatively rapidly (i.e., <1 min), and the ATP synthesis rate rapidly decreased. To allow for quantitative comparisons, information about the time-dependence of ATP synthesis activity, as determined by the biochemical assay and by the dwell time determined by the single-molecule FRET experiment, should be provided.
6. In Fig. 3, only 120- and 36-degree step sizes are considered for the rotation of the c-ring. Although the preferred stoichiometry of c subunits in a ring is reportedly 10 for E. coli ATP synthase (Jiang et al., PNAS 2001; this paper should be referenced), there is another study reporting that the number varies with growth conditions (Schemidt et al., J Bacteriol. 1998). Therefore, the number of c subunits for E. coli ATP synthase is still controversial. The method used in this study has the potential to clinch the decision on this issue. Other models, such as 32.7- (11 c subunits) and 30- (12 c subunits) degree step sizes should be tried and compared.

Minor points
1. In the Materials and Methods section, ATP synthesis and hydrolysis was described as being measured at 25 or 37 degrees Celsius. The temperature of the single-molecule experiments should be also given. Which temperature was applied to the ATP synthesis activity of wild type (27 s-1), as described on page 3?
2. The following papers should be referenced:
Jiang W, Hermolin J, Fillingame RH. The preferred stoichiometry of c subunits in the rotary motor sector of Escherichia coli ATP synthase is 10. Proc Natl Acad Sci U S A. 2001 98:4966-71.
Nishio K, Iwamoto-Kihara A, Yamamoto A, Wada Y, Futai M. Subunit rotation of ATP synthase embedded in membranes: a or beta subunit rotation relative to the c subunit ring. Proc Natl Acad Sci U S A. 2002 99:13448-52. (In Introduction section)
3. There is a typo on page 5, line 8: "20 micrometer" should read as "20 microM".

Referee #3 (Remarks to the Author):

Authors: Monika G. D¸ser, Nawid Zarrabi, Daniel J. Cipriano, Stefan Ernst, Gary D. Glick, Stanley D. Dunn and Michael Boersch.

Title: 36{degree sign} step size of proton-driven c-ring rotation in $F_oF_1$-ATP synthase.

MS #: EMBOJ-2009-70941.

General comments. Building on previous publications, the authors have designed a setup for analysis of the rotation of the c subunit in the rotor of the ATP synthase. They have deduced that the c subunit rotates in steps of 36 degrees. This is an important finding, which illuminates the mechanism of energy-transduction by and transient energy conservation within this exciting biological nanomotor. There appear to be ample data points to back up the conclusions made, and the paper is of outstanding technical novelty and proficiency.

Specific comments.

 

1. page 7 lines 9-10. text needs correction. Is it Fig. 3F?
2. Fig 4 legend last line. correction needed, simulation
3. reference to Zarrabi et al (2009) is incomplete.

Summary recommendation. Accept.

Additional Correspondence from the Authors 12 May 2009

On the manuscript '36{degree sign} step size of proton-driven c-ring rotation in $F_oF_1$-ATP synthase ' by M.G. Dueser et al. submitted to EMBO Journal (EMBOJ-2009-70941)

Statement of referee #2

" The cogency of the conclusion drawn by the authors primarily depends upon the validity of the first analysis procedure, i.e., the manual identification of discrete FRET levels from the time trajectories of the FRET signal. However, this procedure is surprisingly subjective. The four examples of time trajectories from the FRET signal in Fig. 2 show attempts by the authors to identify discrete states in the FRET signal, as denoted by black arrows. However, the authors did not provide any criteria for determining FRET levels, in the midst of such noisy data. How can one agree with the authors' identification? People could easily mark these arrows at different positions, or hesitate to mark at all. Among such noise and largely fluctuating signals, manual identification should not be employed. The verification of discrete FRET levels by objective means is definitely required. Any conclusions drawn from such subjective analysis is not acceptable; thus, this manuscript is not suitable for publication. "

*Initial response to the statement:*

*We absolutely agree with the referee that the key problem of analyzing our single-molecule FRET data in a simple way is the limited signal-to-noise ratio for an unquestionable detection of the short (several milliseconds only) and the multiple stopping positions of the c-ring upon ATP synthesis (5 or 6 between the minimal and maximal FRET distances during a full rotation). This is exactly the reason why we had to solve this problem first before we were convinced to publish the result in the given manuscript. Because we want to make our FRET data analysis progress as clear as possible for the general audience we are grateful for the opportunity to improve our manuscript in a revised form in response to the referee comments.*

*Our very first FRET data of c-ring rotation were presented at the European Bioenergetics Conference in Moscow, 2006 (see M. G. Dueser et al. BIOCHIMICA ET BIOPHYSICA ACTA-BIOENERGETICS Pages: 302 Supplement: Suppl. S). At that time, we had observed a different stepping behavior of the c-ring in contrast to a 120{degree sign} stepping as the reference. We could directly compare the stepping of the ε subunit (120{degree sign} steps in $F_oF_1$) using the same fluorophores, i.e. EGFP on the C-terminus of subunit α as FRET donor, and Alexa568 at ε as the FRET acceptor. This work is published in JBC (Dueser et al., Nov. 2008) and examples of three distinguished FRET levels are shown there. In this and the other previous 120{degree sign} stepping FRET analyses, manual assignment of stopping positions by FRET states was easily possible because the dwell times were long (> 10 ms) and, accordingly, the number of photons used to define a FRET level and the transitions between them were high. The two-dimensional FRET transition density plot showed three well-separated populations (i.e. FRET state transitions 1->2, 2->3, and 3->1) and the direction of rotation changed from ATP hydrolysis to ATP synthesis conditions.*

*In the first c-ring rotation FRET data 2006 we could not identify a set of three FRET states anymore. On the other hand, we also could not clearly identify simple series of 5 or more consecutive 36{degree sign} steps, but we noticed that sometimes short FRET levels seemed to occur (examples are shown in Figure 2 A, B of our EMBO J manuscript). In addition, the published c-ring rotation data upon ATP hydrolysis using detergent-solubilized $F_oF_1$ from the thermophilic PS3 (Ueno at al., PNAS 2005) suggested 120{degree sign} stepping like those authors have found for the gamma subunit.*

 

*So, we did not know how many FRET states we have to expect for the c-ring rotation and how long the dwell times are. How to solve this problem?*

*We have developed a set of multiple controls:*

*1) We made different single-molecule FRET measurements using continuous-wave or pulsed laser excitation, and we used different FRET acceptor fluorophores to control the FRET donor photophysics and to exclude photophysical artifacts (using the new alternating laser scheme DCO-ALEX). Thus very large single-molecule FRET data sets were acquired. In addition, the FRET efficiencies of assigned FRET levels could be compared using either fluorescence intensities or FRET donor fluorescence lifetime (Fig. S4). Both ways to calculate FRET efficiencies were found to be strictly correlated.*

*2) Three of the authors did independent manual data analysis on the two large data sets (i.e. continuous-wave and pulsed laser excitation). One of them had no experience with any single-molecule FRET level assignment before. Thus we avoided a single-person-based biased marking of FRET levels. Manual FRET level assignments were done using the standard 1-ms binning as time intervals for the fluorescence time trajectories.*

*3) To compare these FRET analyses we applied the FRET transition density plot as the key component. Taekjip Ha had shown that this is the appropriate approach to identify multiple FRET states, i.e. for more than 3 FRET efficiencies (see reference McKinney SA, Joo C, Ha T (2006) Biophys J 91: 1941-1951). As our three FRET data analysts may have marked FRET levels differently, objective criteria are required to compare theirs results afterwards and to use only unequivocally assigned states. We have applied threshold criteria to all marked FRET levels before including them into the FRET transition density plot as shown in Fig 3F.*
*Each included FRET level had to last for 4 ms at least, had to have more than 50 photons and less than 1000 photons in FRET donor and acceptor channel in total, and the standard deviation of its proximity factor P had to be smaller than 0.15. Similar criteria were applied in our previous studies. The standard deviation limit excludes any marked FRET levels that contained several FRET states with distinct FRET efficiencies. Thus we think that the FRET transition density plot contains only appropriate FRET levels and FRET transitions. As a consequence, the marking of FRET levels within each photon bursts is not always continuous anymore, i.e. there are parts (or time gaps, respectively) within photon bursts where no FRET levels could be assigned or where apparent FRET levels were removed afterwards by those thresholds. The reasons could be either too short FRET states, or too low fluorescence intensities.*

*As pointed out by the referee, we have to include these threshold criteria explicitly in the Methods section, chapter 3.1, of our revised manuscript. In the submitted version this important technical information was missing.*

*4) As the FRET transition density plots were very similar for all three FRET analyses, the next step was to find a way to analyze the plots for making a statement about the step size of the c-ring rotation. The simulation of the expected FRET transition distributions according to 120{degree sign} stepping or to 36{degree sign} stepping was one approach, and comparison with the experimental data clearly indicated the existence of small steps in the range of 36{degree sign}. However, we searched for another independent approach and developed the novel FRET distance change histogram in Fig 4. This data analysis also supports the existence of small step sizes and multiples of these 36{degree sign} steps. The reason for multiple steps is a consequence of the threshold criteria outlined above, which results in missing short steps.*
*We admit that in Fig. 4 the simulation for the possible 40/80{degree sign} sub steps (as observed for the PS3 enzyme) is missing. We will do this simulation for the revised version of the manuscript and will add the comparison / discussion parts.*

*5) The best way to prove that we are not 'trapped by photophysical artifacts' is to change the biochemical conditions for the FRET-based rotation measurements. Therefore we used aurovertin B as a non-competitive inhibitor and compared FRET data upon ATP hydrolysis with those upon ATP synthesis. In our study we used this inhibitor instead of measuring the idling motion of c in the absence of nucleotides, because we wanted to have a chance of observing rotation and to avoid the problem of FRET efficiency fluctuations caused by EGFP photophysics. For the c idling motion in*

 

*the absence of a driving force we are currently developing a similar FRET experiment but will use a trap for the proteoliposome to prolong observation time (which will allow for other fluctuation analysis methods).*
*Doing the same FRET analysis procedures as described above, we obtained clear evidence that aurovertin B slows down the rotational speed during ATP hydrolysis, and that aurovertin is able to block rotation more efficiently during ATP synthesis (Fig. 2E, F and S5). These measurements are independent from the single-molecule FRET aurovertin measurements which were submitted to Biochemistry and which are in revision right now (if required we will provide the revised manuscript).*

*6) After ensuring that we have obtained confidence about the 36{degree sign} step size of proton-driven c ring rotation the last problem arose how to visualize the multi-stepped rotation in examples of single photon bursts. As stated above, our threshold criteria to identify a FRET level are based on minimum photon count rates. Manual FRET level assignments were done using the standard 1-ms binning as time intervals as we did in all our previous studies. However, with this 1-ms binning interval FRET levels shorter than 4 ms cannot be found and not be shown. Therefore we implemented the sophisticated "sliding time window FRET analysis" as suggested by the Claus Seidel group and explained in detail (Margittai et al. PNAS 2003). With their approach, the time resolution can be arbitrarily set to 100 microseconds or even faster using a photon-by-photon calculation of the likely FRET distance. As a consequence, short states become clearly visible because they consist of many data points with 0.1 ms resolution (but the confidence interval or the width of the FRET distance still depends on the photon count rate). We chose this visualization method for Fig. 2 A-D and will provide additional photon bursts in the revised Supplementary information. We have to make clear in the manuscript that the "sliding time window FRET analysis" was used only to visualize the short FRET states in the given examples, but for the assignment of FRET levels and for the threshold-corrected FRET transition density plot we did not use this sophisticated method. We agree with the referee that the FRET distance trajectories apparently look noisy, but we believe that this is the best way to show the existence of short FRET states. As suggested also by referee #1, we will try to improve the perceptibility of the FRET levels by adding horizontal lines to Fig. 2.*

*In summary, we hope that we could provide evidence that our single-molecule FRET data analysis methods and controls are objective, sophisticated, and are appropriate for the detection of short FRET states which is at the limit of current single-molecule FRET analysis methods. In the revised manuscript we will clarify the applied procedures and add these informations as indicated here. Fluctuation analysis methods like burst wise cross-correlation of FRET donor and acceptor channels are not suitable here due to the limited burst lengths and the multiple FRET levels. In another study we have used this burst wise cross-correlation analysis to support the even faster dynamics of the membrane-embedded $K^+$ transporter Kdp where only two main FRET states occurred.*
*Software-based FRET level search algorithms (i.e. the Hidden Markov models we developed to analyze the 120{degree sign} stepping motion of γ and ε in $F_oF_1$) fail for this type of FRET data on c-ring rotation. To the best of our knowledge there is no alternative available if one wants to use freely diffusing proteoliposomes to avoid the artifact induced by surface attachment.*

Major points

" 1. The donor-acceptor distance was calculated from FRET, assuming the isotropic rotation (or distribution) of the fluorophore of EGFP. However, the tethering of EGFP to the C-terminal of a subunit would restrict or bias the rotation of EGFP. The authors may claim an apparently low EGFP anisotropy; however, they should note that this does not guarantee the isotropic distribution of the angle of the EGFP. "

*response*

*The orientation factor $\kappa^2$ affects the accuracy of distance measurements by FRET. The comparatively high anisotropy of the EGFP fluorophore is caused by a rigid fluorophore environment within the autofluorescent protein in combination with a short fluorescence lifetime. Therefore we have carefully measured the distribution of the single-molecule fluorescence anisotropy values of both fluorophores EGFP and Alexa568 when attached to $F_oF_1$-ATP synthase in*

 

*a liposome. We find high anisotropy values around 0.3 but we did not find a higher anisotropy for the $F_oF_1$-tethered EGFP than for EGFP freely diffusing in solution (see Seidel and coworkers, J Phys Chem A 1998, 103, 331-336). The histograms are shown in the supplementary information. Thus the reader is enabled to estimate the error limits of our FRET distance measurements, for instance using the approach given by Dale, Eisinger and Blumberg (Biophysical Journal 1979, 26, p. 161-194). According to their calculations, the distance error due to our measured fluorophore anisotropies affecting $\kappa^2$ is much less than 20 %.*
*We have checked the FRET distance error in the simulations for Fig. 4. Using a standard error of 0.1 nm we could not reproduce the experimental FRET distance change histograms. However, the assumption of a 10% error for each FRET level (corresponding to about 0.5 nm) was the appropriate choice to reprodude the FRET distance change histograms. This information will be added and discussed explicitely in the revised version of the manuscript.*
*Deviations from the mean orientation factor $\kappa^2$ will also affect the FRET transition density plot (Fig. 3F) and will result in a broadening of the transitions. Using the Monte Carlo simulations for Fig. 4 we could generate the FRET transition density plots. Applying a standard error of 0.1 nm failed to reproduce the experimental data. Instead, the standard error or 0.5 nm was neccessary (Fig. 4I). We have found a broadening which we attributed to the low quantum yield of our EGFP construct fused to the ATP synthase. However, we will add the $\kappa^2$ argument to the revised manuscript.*

" 2. It is not at all clear, how the authors deduced the unidirectional rotation from the given trajectories of the FRET signal, which does not provide information regarding a single turn of the c10-ring. A random rotation with a 36-degree step is also able to reproduce the data. Have the authors carried out the requisite control experiment, without a proton motive force? The findings of such an experiment should be provided, to discriminate experimentally the unidirectional and random motions. "

*response*

*We have measured c-ring rotation at various biochemical conditions and we will add the requested statistical information to the revised manuscript. For example, in the presence of AMPPNP we found about 2.5% of FRET-labeled ATP synthases showing 2 or more FRET levels in a photon burst. In contrast, in the presence of ATP (i.e. upon ATP hydrolysis) 17.9% of the ATP synthases showed 2 or more FRET levels in a photon burst. However, these FRET data were obtained using pulsed alternating excitation and the FRET data sets are much smaller than the FRET data set used for the FRET transition density plot upon ATP synthesis condition.*
*Using the large ATP synthesis FRET data set, unidirectional motion of the c ring was identified by two subsequent steps from one distance zone to the next as depicted in Fig. 3B (zone 1 2 3 ... ) and was written in the manuscript on page 8. However, we will clarify this approach to identify unidirectional motion in the revised manuscript. In contrast, we have attributed a random motion ("idling") of the c ring to oscillations between adjacent distance zones. We will add the percentage of FRET transitions attributed to this "idling" motion.*
*This important idling movements will be investigated in future experiments using improved fluorophores and a novel method to trap single proteoliposomes in solution. Thereby we hope to get higher accuracy for the FRET distance determination as well as long observation times for several full rotations under various catalytic conditions. Here, we focus on the discrimination of 120{degree sign} steps versus 36{degree sign} steps during ATP synthesis.*

" 3. Taking previous studies into account, it is strange that Fo makes steps of 36 degrees each, in 13 ms, in the ATP hydrolysis. Woodbury's group determined the velocity of proton translocation to be 3,100 protons/s (Franklin et al., Biophysical Journal 2004). This means that the waiting time of Fo for each proton translocation is only 0.3ms, too short to be detected. This finding is consistent with the Yoshida group's report (Ueno et al., PNAS 2005), that Fo neither affects nor limits rotation; they observed 120-degree stepping rotation of Fo but not any small substep in the ATP-driven rotation. Thus, there is a serious discrepancy between this study's finding of a 36-degree step in hydrolysis and the findings of previous studies. An explanation to this effect should be provided. "

*response*

*As we detect single-molecules here, the comparison with biochemical ensemble results like proton transport rates is difficult. The data published in Biophys. J. 2004 focus on $F_o$ parts without $F_1$*

*reconstituted into liposomes. Thus a comparison with our coupled $F_oF_1$ holoenzyme is not possible. The ATP hydrolysis data shown in our manuscript as supplemental information comprise several step sizes but mostly large steps as seen in the FRET transition density plot Fig. S5C (corresponding to 108{degree sign} or 144{degree sign} step sizes). Therefore the c-ring dwell times are in good agreement with our γ and ε rotation FRET data with the same fluorophores (Dueser et al. JBC 2008). Our previous single-molecule FRET measurements of the γ or ε rotation in reconstituted $F_oF_1$ have revealed mean dwell times between 9 ms and 19 ms for the 120{degree sign} step in the $F_1$ motor during ATP hydrolysis. Therefore we expect slightly shorter dwell times for the c-ring rotation during ATP synthesis assuming mainly 36{degree sign} steps (and sometimes multiples of 36{degree sign} steps). Eventually our proteoliposome preparation with small diameters of about 100 to 150 nm might cause different proton transport rates and rotational speeds.*

*We have also measured proton-driven c-ring rotation in single $F_o$ reconstituted into liposomes but with a limited and smaller FRET data set which is not directly comparable to the ATP synthesis data set. We found stepwise rotation of the c-ring (M.G. Dueser, PhD thesis, University of Freiburg, 2008, in german). However, the FRET transition density plot did show additional small transitions (i.e. < 36{degree sign}). The relative amount of alternating or oscillating steps was higher. A preliminary dwell time analysis yielded a mean dwell time of ~ 10 ms. We will add a short description of these data to the revised manuscript.*
*Because we currently develop a new and improved single-molecule FRET approach to measure the idling motion of the c subunits in the absence of any driving force, FRET data with reconstituted Fo will be re-measured as one of the necessary controls, and these will be presented in the future.*

*Regarding the Ueno et al. paper we think that their measurement conditions are different using a detergent-solubilized $F_oF_1$ from a thermophilic bacterium*

"4. Another discrepancy is the torsion energy in the rotor complex, due to the difference in rotational step size between $F_o$ and $F_1$. The authors claim in this work that $F_o$ makes 36-degree steps, while they previously reported that the $F_1$ portion undergoes a 120-degree stepping rotation in the whole complex of ATP synthase, even in ATP synthesis. If this is the case, it means that the rotor part is transiently distorted up to 84 degrees. How does the rotor complex store such large torsion? Jungle's group reported the stiffness of the parts of the rotor, which should store the torsion if it happens to be 68 pan/radian. This means that the backward torque exerted by the rotor should be -100 pNnm. It is apparently impossible for the Fo motor to overcome such a large energy gap. "

*response*

*Our finding of a 36{degree sign} step size for the c ring in contrast to the coupled 120{degree sign} stepping indeed causes the important question for the location of the transient elastic energy storage either within the rotor subunits and / or parts of the stator. The single-molecule FRET approach is capable to address this question as we plan to map the rotary step size at various positions. The previously used positions in γ and ε are far away from the N-terminal position in the c subunit on the other side of the membrane used here.*
*We can speculate that eventually the stiffness measurements with detergent-solubilized enzymes in the absence of a lipid bilayer and without a proton gradient and without a electric potential difference might results in a different elastic behavior. Therefore, we hope that our FRET data will stimulate theoreticians to develop new models as well as experimentalists to suggest novel measurements to solve this apparent discrepancy.*

5. In Figs. 2E and 2F, the distributions of dwell time are shown. However, the ATP synthesis rate strongly depends on the amplitude of the proton motive force, which dissipates with time.
Under the experimental conditions used, for how long was the proton motive force sustained? In previous studies, including two referenced by the authors (i.e., Steigmillar et al., PNAS 2008 and Fischer et al., FEBS Lett 1999), the proton motive force dissipated relatively rapidly (i.e., <1 min), and the ATP synthesis rate rapidly decreased. To allow for quantitative comparisons, information about the time-dependence of ATP synthesis activity, as determined by the biochemical assay and by the dwell time determined by the single-molecule FRET experiment, should be provided.

*response*

 

*The proton motif force dissipates quickly after the pH jump is generated by buffer mixing. The ensemble data from Peter Graeber's group analyze the differential gradient of the ATP production driven by the proton motif force. The largest slope of ATP production is at time 0 immediately after the buffer mixing. However, even after 1 minute ATP synthesis continues but at much lower rates. We noticed that the number of rotating ATP synthases in the single-molecule FRET measurements was neglectable after about 5 to 8 minutes. This information will be added in detail to the revised manuscript.*

6. In Fig. 3, only 120- and 36-degree step sizes are considered for the rotation of the c-ring. Although the preferred stoichiometry of c subunits in a ring is reportedly 10 for E. coli ATP synthase (Jiang et al., PNAS 2001; this paper should be referenced), there is another study reporting that the number varies with growth conditions (Schemidt et al., J Bacteriol. 1998). Therefore, the number of c subunits for E. coli ATP synthase is still controversial. The method used in this study has the potential to clinch the decision on this issue. Other models, such as 32.7- (11 c subunits) and 30- (12 c subunits) degree step sizes should be tried and compared.

*response*

*This is very good suggestion. We collaborate with Dr. Gabriele Deckers-Hebestreit (University of Osnabrueck) who co-authored the 1998 paper on apparently variable c-ring composition in E. coli ATP synthases. In their most recent work (Ballhausen et al, J. Bacteriol. 2009) they found a consistent and constant c10-ring stoichiometry for different growth conditions. It seems to be that the c-ring size is not varying. This argument will be discussed and both references will be added to the revised manuscript.*

*Instead we look for an alternative way to measure c-ring rotation in ATP synthases with different ring sizes. We collaborate with Greg Cook (University of Otago, Dunedin, New Zealand) who has produced a $F_oF_1$ with a c-ring comprising 13 subunits. Rotation studies of the c ring in this $F_oF_1$-ATP synthase will be started soon using an improved FRET labeling strategy at subunits a and c. In preliminary work we could already observe FRET distances changes due to c-ring rotation. However, it is not yet clear to us whether we achieved the necessary increase in photon count rates to discriminate the 13-stepped rotation from a 10-stepped rotation.*

Minor points

1. In the Materials and Methods section, ATP synthesis and hydrolysis was described as being measured at 25 or 37 degrees Celsius. The temperature of the single-molecule experiments should be also given. Which temperature was applied to the ATP synthesis activity of wild type (27 s-1), as described on page 3?

*The single-molecule measurements were done at room temperature at 20 {degree sign}C. Ensemble ATP synthesis rates for the mutant and a preparation of the wild type enzyme were measured in parallel at 25{degree sign} C in the Graeber laboratory in Freiburg. The wild type ATP synthesis rates were in perfect agreement to our previous measurements under the same experimental conditions (see EMBO J 2005). This information will be added to the revised manuscript.*

2. The following papers should be referenced: Jiang W, Hermolin J, Fillingame RH. The preferred stoichiometry of c subunits in the rotary motor sector of Escherichia coli ATP synthase is 10. Proc Natl Acad Sci U S A. 2001 98:4966-71. Nishio K, Iwamoto-Kihara A, Yamamoto A, Wada Y, Futai M. Subunit rotation of ATP synthase embedded in membranes: a or beta subunit rotation relative to the c subunit ring. Proc Natl Acad Sci U S A. 2002 99:13448-52. (In Introduction section)

*The references were missing and will be added in the revision.*

3. There is a typo on page 5, line 8: "20 micrometer" should read as "20 microM".

*This will be corrected.*

 

Additional Correspondence from the Editor 03 June 2009

Thank you for your email. As indicated to you in my previous email, I had asked for further input on your manuscript, the concerns raised by referee #2 and your point-by-point response from a good expert in the field. I have now heard back from the editorial advisor. The advisor appreciates the concerns raised by referee #2, but also finds that you adequately respond to the raised concerns in your point-by-point response. Given this independent assessment, I would like to ask you to submit a suitably revised manuscript that addresses the concerns raised by all three referees and to revise the manuscript along the lines as indicated in your point-by-point response.

1st Revision - authors' response 04 June 2009

Response to the referee's comments and suggestions on the manuscript
'36o step size of proton-driven c-ring rotation in $F_oF_1$-ATP synthase '
by M.G. Dueser et al. submitted to EMBO Journal (EMBOJ-2009-70941)

Referee #1

1) Data analysis: 24% of close to 12,000 molecules had both dyes, 1294 bursts were used for FRET transition density plot and 1879 for distance histograms (page 7). A table summarizing these numbers and where they came from would be helpful.

*This is a very good suggestion. We have added a new paragraph to the Supplementary information where we explain which of the two single-molecule FRET data sets was used for the figures. We carefully re-checked all given numbers and provide the complete statistics in the Supplementary information.*

2) Data interpretation: The placement of the arrows in Fig. 2A and B is not entirely clear. Are the arrows supposed to point to different FRET levels (especially in 2B there are more 'levels' than arrows)? Since these 'bursts' were analyzed manually (as mentioned on page 4, bottom paragraph), maybe include horizontal lines corresponding to the 5 distance bins.

*We use two types of arrows (black and red) in Fig. 2 to indicate two different phenomena.*
*In Fig 2A and B we want to show that a series of small distance changes can be observed during ATP synthesis within a single photon burst. Thus photon bursts were selected that show unidirectional rotation of the c ring, either showing stepwise distance decrease (Fig. 2A) or increase (Fig. 2B). Black arrows indicate discrete FRET levels in the burst, but only those FRET levels that change from the maximum to minimum distance, or vice versa. Red arrows in Figure 2C and D show the level transitions from one to the next FRET level. These transitions were selected to show that we observed larger stepsizes as well where substeps apparently could not be resolved.*

*We have clarified the use of the arrows in the manuscript on page 5 first paragraph, and have added 4 additional examples of photon bursts in the Supplementary information as Fig. S6. there we applied horizontal lines as suggested showing the boundaries for the 5 FRET levels zones (highlighted in yellow).*

3) Page 4, bottom: How do the authors calculate the 36 1/s? A 9 ms dwell results in 111

 

protons/s and assuming 4 protons per ATP would give a turnover number of 28 (very close to the measured 27 1/s).

*Previously we had calculated the single-molecule turnover in a slightly different way using the number of 10 c subunits. Now we have taken the suggested procedure from the referee which is added on page 5 as last sentence. We also added the information about the measurement temperature.*

4) Page 5, top: The effect of Aurovertin B binding is not clear. The number of rotating ATP synthases "...was reduced by 75% and the dwell times were prolonged more than 2-fold". How many molecules had both dyes, how many did rotate? Does aurovertin B binding inhibit 100% (prevent rotation) or only slow down activity? Did the authors measure ATP synthesis biochemically with and without Aurovertin? The authors say that residual activity is 50% "... in agreement with biochemical measurements" but no data are shown or paper is cited. Same question for DCCD inhibition: On page 10 it says that 60 μM DCCD abolishes ATP synthesis. On page 4 is mentions an inhibition efficiency of 63%.

*The discussion around aurovertin B refers to a manuscript by Johnson et al. which is now in press in Biopolymers (DOI: 10.1002/bip.21262) . There, single-molecule FRET data measured between different positions in the rotor (γ subunit in $F_1$) and stator (b subunits in $F^o$) indicate that aurovertin B slows down rotation in the case of ATP hydrolysis, but tends to block rotation in the case of ATP synthesis. As the reader may not be able to access the other information there, we now have added the complete statistical information of the single-molecule FRET data of c-ring rotation to the Supplementary information section. The reference to the paper in Biopolymers will be added as soon as possible during the publication process.*
*In addition we have corrected the statement of DCCD inhibition to "nearly abolishes synthesis" on page 12 line 7. The discrepancy is most likely due to the limited amount of the single-molecule data showing a residual activity.*

5) What could be the source of the discrepancy between observed and simulated FRET transition density plot in terms of probabilities? In the observed plot, most distance changes are seen at the long sides of the ellipse (similar to Fig. S5) whereas in the simulated plot (Fig. 4I), most probability is at the tips.

*This is a very important point. For the experimental data we have applied a threshold of 0.5 nm for the distance difference required to separate two adjacent stopping position. Thus we cannot discriminate very small distance changes; instead these levels will be combined. This results in the loss of FRET transition data points at the tips of the ellipse in the FRET transition density plot, and, in addition, prolongs the mean dwell time in the histograms Fig 2E,F and S5A and B. In contrast, the simulation does not have this threshold for the FRET level assignment and, accordingly, the number of data point at the tips is higher because of the higher density of stopping positions there (see Fig. 3B).*

*We have added this discussion on page 10 line 1 in the manuscript.*

6) Could it be that the 40° substep in gamma/epsilon (e.g. Yasuda et al., 2001) stepping is actually a 36° step of one c subunit (at least in E. coli $F_1F_0$)?

*We have simulated the 40°/80° substep scenario as suggested, and have added the result as a new Figure 4G. The simulated FRET distance change histogram looks similar to the 36° /72° /108° /144° plot (shown now as Fig. 4H). We have added this statement on page 9 line 5. However, if we assume purely 40/80 stepping, the number of 138 detected subsequent 36odouble steps cannot be explained at all. Therefore, we think that the 40/80 scenario is not the right one.*

 

7) Can the authors speculate as to how the coupling can accommodate the requirement of 4 protons/ATP with only 10 c subunits (protons) per revolution?

*At the moment our single-molecule FRET data do not cover several full turns of the cring within a single molecule. This is required to identify repeated step sizes for different orientations of the c-ring more accurately. We are working on a new experimental approach to trap single proteoliposomes during the FRET measurements, but this is work in progress. Therefore we are not able to make a reasonable suggestion like a 3-3-4 scheme or something else based on the current data.*

8) Overall, the manuscript needs careful editing: - there are no page numbers
- page 4, bottom paragraph: Affecting instead of effecting or better 'effect of aurovertin B on dwell time..'

*We have changed the sentence.*

- page 5,middle: Fix the sentence "These effects of ..."

*The sentence has been changed.*

- page 7, end of top paragraph: "... shown as Fig. F the manuscript)" Something seems to be missing here.

*The missing figure number has been added.*

- page 8, middle: "This might be due to limited the..."

*The sentence has been changed.*

- Fig. 3A is not referred to in the text (and the order of the other parts of the figure is mixed up, with F coming first etc.)

*Figure 3A is referred to on page 7 line 3 now, and the order of the parts in Fig. 3 A-F is maintained in the manuscript now. This also supports those readers who want to be guided by reading the figure legends first.*

- Same for Fig. 4. Not all parts are referred to in the text. This makes it harder to follow the description of the results.

*Now, Fig. 4 is described with all parts, and the order of the individual histograms is maintained in the discussion of manuscript on page 9 first paragraph.*

Referee #2

Statement
" The cogency of the conclusion drawn by the authors primarily depends upon the validity of the first analysis procedure, i.e., the manual identification of discrete FRET levels from the time trajectories of the FRET signal. However, this procedure is surprisingly subjective. The four examples of time trajectories from the FRET signal in Fig. 2 show attempts by the authors to identify discrete states in the FRET signal, as denoted by black arrows. However, the authors did not provide any criteria for determining FRET levels, in the midst of such noisy data. How can one agree with the authors' identification? People could easily mark these arrows at different

 

positions, or hesitate to mark at all. Among such noise and largely fluctuating signals, manual identification should not be employed. The verification of discrete FRET levels by objective means is definitely required. Any conclusions drawn from such subjective analysis is not acceptable; thus, this manuscript is not suitable for publication. "

*response to the statement:*

*We absolutely agree with the referee that the key problem of analyzing our singlemolecule FRET data in a simple way is the limited signal-to-noise ratio for an unquestionable detection of the short (several milliseconds only) and the multiple stopping positions of the c-ring upon ATP synthesis (5 or 6 between the minimal and maximal FRET distances during a full rotation). This is exactly the reason why we had to solve this problem first before we were convinced to publish the result in the given manuscript. Because we want to make our FRET data analysis progress as clear as possible for the general audience we are grateful for the opportunity to improve our manuscript in the revised form in response to the referee comments.*

*Our very first FRET data of c-ring rotation were presented at the European Bioenergetics Conference in Moscow, 2006 (see M. G. Dueser et al. BIOCHIMICA ET BIOPHYSICA ACTA-BIOENERGETICS Pages: 302 Supplement: Suppl. S). At that time, we had observed a different stepping behavior of the c-ring in contrast to a $120^o$ stepping as the reference. We could directly compare the stepping of the ε subunit ($120^o$ steps in $F_oF_1$) using the same fluorophores, i.e. EGFP on the C-terminus of subunit a as FRET donor, and Alexa568 at ε as the FRET acceptor. This work is published in JBC (Dueser et al., Nov. 2008) and examples of three distinguished FRET levels are shown there. In this and the other previous $120^o$ stepping FRET analyses, manual assignment of stopping positions by FRET states was easily possible because the dwell times were long (> 10 ms) and, accordingly, the number of photons used to define a FRET level and the transitions between them were high. The two-dimensional FRET transition density plot showed three well-separated populations (i.e. FRET state transitions 1->2, 2->3, and 3->1) and the direction of rotation changed from ATP hydrolysis to ATP synthesis conditions.*

*In the first c-ring rotation FRET data 2006 we could not identify a set of three FRET states anymore. On the other hand, we also could not clearly identify simple series of 5 or more consecutive 36osteps, but we noticed that sometimes short FRET levels seemed to occur (examples are shown in Figure 2 A, B of the manuscript and in the Supplement). In addition, the published c-ring rotation data upon ATP hydrolysis using detergent-solubilized $F_oF_1$ from the thermophilic PS3 (Ueno at al., PNAS 2005) suggested $120^o$ stepping like those authors have found for the γ subunit.*

*So, we did not know how many FRET states we have to expect for the c-ring rotation and how long the dwell times are. How to solve this problem?*

*We have developed a set of multiple controls:*

*1) We made different single-molecule FRET measurements using continuous-wave or pulsed laser excitation, and we used different FRET acceptor fluorophores to control the FRET donor photophysics and to exclude photophysical artifacts using the new alternating laser scheme DCO-ALEX. Thus very large single-molecule FRET data sets were acquired. In addition, the FRET efficiencies of assigned FRET levels could be compared using either fluorescence intensities or FRET donor fluorescence lifetime (Fig. S4). Both ways to calculate FRET efficiencies were found to be strictly correlated.*

*2) Three of the authors did independent manual data analysis on the two large data sets (i.e. continuous-wave and pulsed laser excitation). One of them had no experience with*

 

*any single-molecule FRET level assignment before. Thus we avoided a single-personbased biased marking of FRET levels. Manual FRET level assignments were done using the standard 1-ms binning as time intervals for the fluorescence time trajectories.*

*3) To compare these FRET analyses we applied the FRET transition density plot as the key component. Taekjip Ha had shown that this is the appropriate approach to identify multiple FRET states, i.e. for more than 3 FRET efficiencies (see reference McKinney SA, Joo C, Ha T (2006) Biophys J 91: 1941-1951). As our three FRET data analysts may have marked FRET levels differently, objective criteria are required to compare theirs results afterwards and to use only unequivocally assigned states. We have applied threshold criteria to all marked FRET levels before including them into the FRET transition density plot as shown in Fig 3F.*
*Each included FRET level had to last for 4 ms at least, had to have more than 50 photons and less than 1000 photons in FRET donor and acceptor channel in total, and the standard deviation of its proximity factor P had to be smaller than 0.15. Similar criteria were applied in our previous studies. The standard deviation limit excludes any marked FRET levels that contained several FRET states with distinct FRET efficiencies. Thus we think that the FRET transition density plot contains only appropriate FRET levels and FRET transitions. As a consequence, the marking of FRET levels within each photon bursts is not always continuous anymore, i.e. there are parts (or time gaps, respectively) within photon bursts where no FRET levels could be assigned or where apparent FRET levels were removed afterwards by those thresholds. The reasons could be either too short FRET states, or too low fluorescence intensities.*
*We have included these threshold criteria explicitly in the Methods section, chapter 3.1, of our revised manuscript and in detail in the Supplementary Information.*

*4) As the FRET transition density plots were very similar for all three FRET analyses, the next step was to find a way to analyze the plots for making a statement about the step size of the c-ring rotation. The simulation of the expected FRET transition distributions according to 120$^{o}$ stepping or to 36$^{o}$ stepping was one approach, and comparison with the experimental data clearly indicated the existence of small steps in the range of 36$^{o}$. However, we searched for another independent approach and developed the novel FRET distance change histogram in Fig 4. This data analysis also supports the existence of small step sizes and multiples of these 36$^{o}$ steps. The reason for multiple steps is a consequence of the threshold criteria outlined above, which results in missing short steps.*
*We admit that in the original Fig. 4 the simulation for the possible 40/80$^{o}$ sub steps (as observed for the PS3 enzyme) was missing. We did this simulation for the revised version of the manuscript and have added the comparison.*

*5) The best way to prove that we are not 'trapped by photophysical artifacts' is to change the biochemical conditions for the FRET-based rotation measurements. Therefore we used aurovertin B as a non-competitive inhibitor and compared FRET data upon ATP hydrolysis with those upon ATP synthesis. In our study we used this inhibitor instead of measuring the idling motion of c in the absence of nucleotides, because we wanted to have a chance of observing rotation and to avoid the problem of FRET efficiency fluctuations caused by EGFP photophysics. For the c idling motion in the absence of a driving force we are currently developing a similar FRET experiment but will use a trap for the proteoliposome to prolong observation time (which will allow for other fluctuation analysis methods).*
*Doing the same FRET analysis procedures as described above, we obtained clear evidence that aurovertin B slows down the rotational speed during ATP hydrolysis, and that aurovertin is able to block rotation more efficiently during ATP synthesis (Fig. 2E, F and S5). These measurements are independent from the single-molecule FRET aurovertin measurements which are published now in Biopolymers.*

*6) After ensuring that we have obtained confidence about the 36$^{o}$ step size of protondriven c ring rotation the last problem arose how to visualize the multi-stepped rotation in examples of single photon bursts. As stated above, our threshold criteria to identify a*

 

*FRET level are based on minimum photon count rates. Manual FRET level assignments were done using the standard 1-ms binning as time intervals as we did in all our previous studies. However, with this 1-ms binning interval FRET levels shorter than 4 ms cannot be found and not be shown. Therefore we implemented the sophisticated "sliding time window FRET analysis" as suggested by the Claus Seidel group and explained in detail (Margittai et al. PNAS 2003). With their approach, the time resolution can be arbitrarily set to 100 microseconds or even faster using a photon-by-photon calculation of the likely FRET distance. As a consequence, short states become clearly visible because they consist of many data points with 0.1 ms resolution (but the confidence interval or the width of the FRET distance still depends on the photon count rate). We chose this visualization method for Fig. 2 A-D and provide additional photon bursts in the revised Supplementary information. We explained in the revised manuscript that the "sliding time window FRET analysis" was used only to visualize the short FRET states in the given examples, but for the assignment of FRET levels and for the thresholdcorrected FRET transition density plot we did not use this sophisticated method. We agree with the referee that the FRET distance trajectories apparently look noisy, but we believe that this is the best way to show the existence of short FRET states. As suggested also by referee #1, we tried to improve the perceptibility of the FRET levels by adding horizontal lines to the photon bursts in the Supplementary Information (Fig. S6).*

*In summary, we hope that we could provide evidence that our single-molecule FRET data analysis methods and controls are objective, sophisticated, and are appropriate for the detection of short FRET states which is at the limit of current single-molecule FRET analysis methods. In the revised manuscript we have clarified the applied procedures and add these informations as indicated here.*
*Fluctuation analysis methods like burst wise cross-correlation of FRET donor and acceptor channels are not suitable here due to the limited burst lengths and the multiple FRET levels. In another study we have used this burst wise cross-correlation analysis to support the even faster dynamics of the membrane-embedded K+ transporter Kdp where only two main FRET states occurred.*
*Meanwhile software-based FRET level search algorithms (i.e. the Hidden Markov models we developed to analyze the 120$^o$ stepping motion of and in $F_oF_1$) were developed futher and were appled to the c-ring rotation FRETdata. Given 5 FRET levels with free changable FRET efficiencies and very long dwell times as startuing values resulted in a repoduction of the 36$^o$ stepping behaviour with dwell times betwen 6 an 11 ms. However, this results is too preliminary to be included in the manuscript now. To the best of our knowledge there is no alternative available if one wants to use freely diffusing proteoliposomes to avoid the artifact induced by surface attachment.*

Minor Points

1. The donor-acceptor distance was calculated from FRET, assuming the isotropic rotation (or distribution) of the fluorophore of EGFP. However, the tethering of EGFP to the C-terminal of a subunit would restrict or bias the rotation of EGFP. The authors may claim an apparently low EGFP anisotropy; however, they should note that this does not guarantee the isotropic distribution of the angle of the EGFP.

*The orientation factor $\kappa^2$ affects the accuracy of distance measurements by FRET. The comparatively high anisotropy of the EGFP fluorophore is caused by a rigid fluorophore environment within the autofluorescent protein in combination with a short fluorescence lifetime. Therefore we carefully measured the distribution of the single-molecule fluorescence anisotropy values of both fluorophores EGFP and Alexa568 when attached to $F_oF_1$-ATP synthase in a liposome. We find high anisotropy values around 0.3 but we did not find a higher anisotropy for the $F_oF_1$-tethered EGFP than for EGFP freely diffusing in solution (see Seidel and coworkers, J Phys Chem A 1998, 103, 331-336). The histograms are shown in the supplementary information. Thus the reader is enabled to estimate the error limits of our FRET distance measurements, for instance using the approach given by Dale, Eisinger and Blumberg (Biophysical Journal 1979, 26, p. 161-194). According to their calculations, the distance error due to our measured fluorophore*

*anisotropies affecting $\kappa^2$ is much less than 20 %. This information has been added and discussed on page 13 line 16 and in the Supplementary information.*
*We have checked the FRET distance error in the simulations for Fig. 4. Using a standard error of 0.1 nm we could not reproduce the experimental FRET distance change histograms. However, the assumption of a 10% error for each FRET level (corresponding to about 0.5 nm) was the appropriate choice to reproduce the FRET distance change histograms. This information is discussed on page 8 last paragraph and in the Supplementary information.*
*Deviations from the mean orientation factor $\kappa^2$ will also affect the FRET transition density plot (Fig. 3 F) and will result in a broadening of the transitions. Using the Monte Carlo simulations for Fig. 4 we could generate the FRET transition density plots. Applying a standard error of 0.1 nm failed to reproduce the experimental data. Instead, the standard error of 0.5 nm was needed (Fig. 4 I). We have found a broadening which we attributed to the low quantum yield of our EGFP construct fused to the ATP synthase.*

2. It is not at all clear, how the authors deduced the unidirectional rotation from the given trajectories of the FRET signal, which does not provide information regarding a single turn of the c10-ring. A random rotation with a 36-degree step is also able to reproduce the data. Have the authors carried out the requisite control experiment, without a proton motive force? The findings of such an experiment should be provided, to discriminate experimentally the unidirectional and random motions. For this purpose, simulation data is not required.

*We have added a detailed description of the procedures to get objective FRET level data from the initial manual assignment of FRET levels to the manuscript on page 5 and the Supplementary information. We have measured c-ring rotation at various biochemical conditions and provide the statistical information in the Supplementary Information (Chapter 6) to the revised manuscript.*
*For example, in the presence of AMPPNP we found about 2.5% of FRET-labeled ATP synthases showing 2 or more FRET levels in a photon burst. In contrast, in the presence of ATP (i.e. upon ATP hydrolysis) 17.9% of the ATP synthases showed 2 or more FRET levels in a photon burst. However, these FRET data were obtained using pulsed alternating excitation and the FRET data sets are much smaller than the FRET data set used for the FRET transition density plot under ATP synthesis condition.*
*Using the large ATP synthesis FRET data set, unidirectional motion of the c ring was identified by two subsequent steps from one distance zone to the next as depicted in Fig. 3 B (zone 1 2 3 ... ) and was written in the manuscript on page 8. In contrast, we have attributed a random motion ("idling") of the c ring to oscillations between adjacent distance zones. These important idling movements will be investigated in future experiments using improved fluorophores and a novel method to trap single proteoliposomes in solution. Thereby we hope to get higher accuracy for the FRET distance determination as well as longer observation times for several full rotations under various catalytic conditions. Here, we focus on the discrimination of $120^o$ steps versus $36^o$ steps during ATP synthesis.*

3. Taking previous studies into account, it is strange that Fo makes steps of 36 degrees each, in 13 ms, in the ATP hydrolysis. Woodbury's group determined the velocity of proton translocation to be 3,100 protons/s (Franklin et al., Biophysical Journal 2004). This means that the waiting time of Fo for each proton translocation is only 0.3ms, too short to be detected. This finding is consistent with the Yoshida group's report (Ueno et al., PNAS 2005), that Fo neither affects nor limits rotation; they observed 120-degree stepping rotation of Fo but not any small substep in the ATP-driven rotation. Thus, there is a serious discrepancy between this study's finding of a 36-degree step in hydrolysis and the findings of previous studies. An explanation to this effect should be provided.

*As we detect single-molecules here, the comparison with biochemical ensemble results like proton transport rates is difficult. The data published in Biophys. J. 2004 focus on the $F_o$ sector without $F_1$ reconstituted into liposomes. Thus a comparison with our coupled $F_oF_1$ holoenzyme is not possible. The ATP hydrolysis data are shown in our manuscript as Supplemental Information. The FRET data set is smaller than the one for ATP*

 

*synthesis conditions. It comprises several step sizes but mostly large steps as seen in the FRET transition density plot Fig. S5C (corresponding to 108° or 144° step sizes, or 120° steps). The relative amount of 36° steps was only 23 %, far less that the 48% found during ATP synthesis. Accordingly, the c-ring dwell times are in good agreement with our previous γ and ε rotation FRET data with the same fluorophores (Dueser et al. JBC 2008). Our earlier single-molecule FRET measurements of the γ or ε rotation in reconstituted $F_oF_1$ have revealed mean dwell times between 9 ms and 19 ms for the 120° step in the $F_1$ motor during ATP hydrolysis. An important difference from the study published in Biophys. J. 2004 is that our proteoliposome preparation with small diameters of about 100 to 150 nm might cause different proton transport rates and rotational speeds because of the build up of a counteracting proton motive force during ATP hydrolysis. For ATP synthesis we expect slightly shorter dwell times for the c-ring rotation assuming mainly 36° steps (and sometimes multiples of 36° steps). Using single Na+-driven ATP synthases from Propionigenium modestum reconstituted into liposomes, Dimroth and coworkers had measured rotational speeds between 12.5 to 50 s-1 per full rotation of the c-ring consisting of multiple steps (FEBS lett. 2002) which corresponds to our dwell times.*

*We have also measured proton-driven c-ring rotation in single Fo reconstituted into liposomes but with a limited and smaller FRET data set which is not directly comparable to the ATP synthesis data set. We found stepwise rotation of the c-ring (M.G. D¸ser, PhD thesis, University of Freiburg, 2008, in German). However, the FRET transition density plot did show additional small transitions (i.e. < 36°). The relative amount of alternating or oscillating steps was higher. A preliminary dwell time analysis yielded a mean dwell time of ~ 10 ms. Because we currently are developing a new and improved single-molecule FRET approach to measure the idling motion of the c subunits in the absence of any driving force, FRET data with reconstituted Fo will be re-measured as one of the necessary controls, and these will be presented in the future.*

*Regarding the Ueno et al. paper their measurement conditions are different, as they used a detergent-solubilized $F_oF_1$ from a thermophilic bacterium.*

4. Another discrepancy is the torsion energy in the rotor complex, due to the difference in rotational step size between $F_o$ and $F_1$. The authors claim in this work that Fo makes 36-degree steps, while they previously reported that the $F_1$ portion undergoes a 120-degree stepping rotation in the whole complex of ATP synthase, even in ATP synthesis. If this is the case, it means that the rotor part is transiently distorted up to 84 degrees. How does the rotor complex store such large torsion? Junge's group reported the stiffness of the parts of the rotor, which should store the torsion if it happens to be 68 pNnn/radian. This means that the backward torque exerted by the rotor should be -100 pNnm. It is apparently impossible for the Fo motor to overcome such a large energy gap.

*Our finding of a 36° step size for the c ring in contrast to the coupled 120° stepping indeed raises the important question of the localization of the transient elastic energy storage either within the rotor subunits and/or parts of the stator. The single-molecule FRET approach is able to address this question as we plan to map the rotary step size at various positions. The previously used positions for the markers in and are far away from the N-terminal position in the c subunit on the other side of the membrane used here.*

*The biophysical aspect of conformational distortion is important and has to be addressed quantitatively as shown by Junge and coworkers (Biophys. J 2008, PNAS 2008, Nature 2009). In their work using surface-attached E. coli $F_oF_1$, the distributions of relative c-ring orientations labeled with short fluorescent actin filaments are broadened with full-widthhalf-maximum about 40° (i.e. ± 20°). However, the tails of the distributions also reach 80∞ but with very low probabilities. This could be taken as a qualitative argument that large angular distortions within the rotor subunits are indeed possible. However, our FRET data are not intended to address this mechanochemical question but we refer to*

 

*the ongoing development of a more sophisticated experiment with the trapped proteoliposomes.*
*We can speculate that the elastic behavior of the detergent-solubilized enzymes in the absence of a lipid bilayer and without a proton gradient or an electric potential difference may differ from the behavior of the enzyme in a proteoliposome with a proton motive force. Therefore, we hope that our FRET data will stimulate theoreticians to develop new models as well as experimentalists to suggest novel measurements to solve this apparent discrepancy.*

5. In Figs. 2E and 2F, the distributions of dwell time are shown. However, the ATP synthesis rate strongly depends on the amplitude of the proton motive force, which dissipates with time. Under the experimental conditions used, for how long was the proton motive force sustained? In previous studies, including two referenced by the authors (i.e., Steigmillar et al., PNAS 2008 and Fischer et al., FEBS Lett 1999), the proton motive force dissipated relatively rapidly (i.e., <1 min), and the ATP synthesis rate rapidly decreased. To allow for quantitative comparisons, information about the time-dependence of ATP synthesis activity, as determined by the biochemical assay and by the dwell time determined by the single-molecule FRET experiment, should be provided.

*The proton motive force dissipates quickly after the pH jump is generated by buffer mixing. The ensemble data from Peter Gr‰ber's group (PNAS 2008, EMBO J 2005) analyze the differential gradient of the ATP production driven by the proton motive force. The largest rate of ATP production is at time 0 immediately after the buffer mixing. However, even after 1 minute ATP synthesis continues but at much lower rates. We noticed that the number of rotating ATP synthases in the single-molecule FRET measurements was negligible after about 5 to 8 minutes. This information has been added in detail to the revised manuscript on page 4 line 2.*

6. In Fig. 3, only 120- and 36-degree step sizes are considered for the rotation of the c-ring. Although the preferred stoichiometry of c subunits in a ring is reportedly 10 for E. coli ATP synthase (Jiang et al., PNAS 2001; this paper should be referenced), there is another study reporting that the number varies with growth conditions (Schemidt et al., J Bacteriol. 1998). Therefore, the number of c subunits for E. coli ATP synthase is still controversial. The method used in this study has the potential to clinch the decision on this issue. Other models, such as 32.7- (11 c subunits) and 30- (12 c subunits) degree step sizes should be tried and compared.

*This is very good suggestion. We collaborate with Dr. Gabriele Deckers-Hebestreit (University of Osnabrueck) who co-authored the 1998 paper on apparently variable c-ring composition in E. coli ATP synthases. In their most recent work (Ballhausen et al, J. Bacteriol. 2009) they found a consistent and constant c10-ring stoichiometry for different growth conditions. It seems to be that the c-ring size is not varying. The new reference has been added to the revised manuscript.*

*Instead we look for an alternative way to measure c-ring rotation in ATP synthases with different ring sizes. We collaborate with Greg Cook (University of Otago, Dunedin, New Zealand) who has produced $F_oF_1$ with a c-ring comprising 13 subunits. Rotation studies of the c-ring in this $F_oF_1$-ATP synthase will be started soon using an improved FRET labeling strategy at subunits a and c. In preliminary work we could already observe FRET distance changes due to c-ring rotation. However, it is not yet clear to us, whether we achieved the necessary increase in photon count rates to discriminate the 13-stepped rotation from a 10-stepped rotation by single-molecule FRET.*

Minor points

1. In the Materials and Methods section, ATP synthesis and hydrolysis was described as being measured at 25 or 37 degrees Celsius. The temperature of the single-molecule experiments

 

should be also given. Which temperature was applied to the ATP synthesis activity of wild type (27 s-1), as described on page 3?

*We have added the required temperature information in the manuscript.*

2. The following papers should be referenced: Jiang W, Hermolin J, Fillingame RH. The preferred stoichiometry of c subunits in the rotary motor sector of Escherichia coli ATP synthase is 10. Proc Natl Acad Sci U S A. 2001 98:4966-71. Nishio K, Iwamoto-Kihara A, Yamamoto A, Wada Y, Futai M. Subunit rotation of ATP synthase embedded in membranes: a or beta subunit rotation relative to the c subunit ring. Proc Natl Acad Sci U S A. 2002 99:13448-52. (In Introduction section)

*These papers have been added as suggested.*

3. There is a typo on page 5, line 8: "20 micrometer" should read as "20 microM".

*This has been changed.*

Referee #3

1. page 7 lines 9-10. text needs correction. Is it Fig. 3F?

*This has been changed.*

2. Fig 4 legend last line. correction needed, simulation

*This has been changed.*

3. reference to Zarrabi et al (2009) is incomplete.

*This has been changed.*

2nd Editorial Decision 26 June 2009

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referee #1 and the editorial advisor (referee #4) to review the revised version and I have now heard back from both. I am pleased to say that both referees support publication here. Referee #4 has a few specific comments that I would like to ask you to take into consideration in a final round of 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.

Thank you for submitting your interesting study to the EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

I feel the authors have addressed all concerns from the first round of review in a satisfactory manner

 

and I support publication of the revised version of the manuscript in EMBO J without further revisions.

Referee #4 (Remarks to the Author):

The authors aimed at the stepped c-ring rotation during ATP synthesis using FRET. Their data are almost submerging in noise, such that hand-picking of discrete FRET-levels could not be avoided. If one did not expect 36{degree sign}-stepping based on structural data of the E. coli enzyme, the manuscript might appear as not acceptable. On the other hand one has to acknowledge that the procedures, controls and caveats presented by the authors (including their response to referees and the supplementary material) are state of the art and merit publication. There are a few specific comments they might consider in a minor revision of this second version.

Specific comments:

1) Fluctuating intensities in the photo bursts (Fig.1C) result from both, the translational AND the rotational diffusion of a vesicle. Because the rotational correlation time is in the time domain of milliseconds, and because both chromophores do not rotate isotropically in the enzyme, this might affect the apparent FRET level even if the c-ring did not rotate.

2) Confidence in their data evaluation relies heavily on the raw data given in Fig. 2A-D. The positioning of the black arrow is debatable. It would serve the manuscript well to present an enlarged version of e.g. the upper panels of Fig. 2AB (Fig. 2E&F might go into the Supplement then), and to enable the reader to check the given choice for placing the black arrows. It might also be explained why some of the sublevels (e.g. in Fig.2B) were not pinpointed by arrows.

3) Feniouk et al. (2004) BPJ 86,4094 and Franklin et al. (2004) BPJ 87, 3594 (both not cited) determined turnover numbers of "free" FO, 6300 and 3000 H+s-1, respectively, at a driving force of 100 mV, implying dwells of 160 and 330 µs. The longer duration of the dwells in the present work suggests rate limitation by the load of ATP-synthesis, if so, one expects that successive steps should have discernibly increasing dwell times, starting from the shortest one right after discharge of ATP towards the last just before, thereby following the winding-up of the elastic element between $F_o$ and $F_1$. Has there been any indication for such a behavior?

p.5,middle "Effecting dwell...." should read 'Affecting .....'
p.7,11 "...white curve ...." There is no white curve in Fig. 3.
Legend Fig.1 color coding of curves not given

2nd Revision - authors' response 29 June 2009

Specific response to the referee's comments and suggestions on the manuscript
'36$^o$ step size of proton-driven c-ring rotation in $F_oF_1$-ATP synthase'
by M.G. Dueser et al. submitted to EMBO Journal (EMBOJ-2009-70941R)

Referee # 4

Specific comments:

1) Fluctuating intensities in the photon bursts (Fig.1C) result from both, the translational AND the rotational diffusion of a vesicle. Because the rotational correlation time is in the time domain of milliseconds, and because both chromophores do not rotate isotropically in the enzyme, this might affect the apparent FRET level even if the c-ring did not rotate.

*The rotational correlation time $\theta=\eta V/kT$ of a vesicle with 100 nm radius in water at 20$^o$C is calculated to ~ 1 ms and might affect fluorescence excitation and detection efficiencies resulting in intensity fluctuations. In addition, the rotational correlation time of the $F_oF_1$-*

*ATP synthase within the lipid membrane is in the s range at room temperature (Musier-Forsyth and Hammes, Biochemistry 1990), i.e. is faster than our time resolution with 1 ms binning and, accordingly, fluorescence excitation anisotropy of the FRET donor is likely to be averaged.*

*We have added on page 3 line 22:*

*"Additionally, the rotational motion of the vesicles in the millisecond time range affects fluorescence excitation and detection efficiencies."*

2) Confidence in their data evaluation relies heavily on the raw data given in Fig. 2A-D. The positioning of the black arrow is debatable. It would serve the manuscript well to present an enlarged version of e.g. the upper panels of Fig. 2AB (Fig. 2E&F might go into the Supplement then), and to enable the reader to check the given choice for placing the black arrows. It might also be explained why some of the sublevels (e.g. in Fig.2B) were not pinpointed by arrows.

*We have chosen the two photon bursts in Figure 2 A and B showing a multi-stepped FRET efficiency, that is, clearly more than 3 short states in the range from minimum to maximum distances. This has been described in the revised version of the manuscript on page 5 line 3. In contrast, the photon bursts in Figure 2 C and D were chosen to show apparently larger step sizes, which we would like to document as a control for the reader. Indeed, the signal-to-noise ratio is limited by the use of EGFP as FRET donor, so that our threshold-based judgment of FRET level assignment was absolutely necessary to obtain reliable FRET distances from the manually marked levels. We expect that the bursts in Figure 2 will be reproduced in a large format to be able to see the details.*

3) Feniouk et al. (2004) BPJ 86,4094 and Franklin et al. (2004) BPJ 87, 3594 (both not cited) determined turnover numbers of "free" FO, 6300 and 3000 H+s-1, respectively, at a driving force of 100 mV, implying dwells of 160 and 330 μs. The longer duration of the dwells in the present work suggests rate limitation by the load of ATP-synthesis, if so, one expects that successive steps should have discernibly increasing dwell times, starting from the shortest one right after discharge of ATP towards the last just before, thereby following the winding-up of the elastic element between $F_o$ and $F_1$. Has there been any indication for such a behavior?

*In the presented version of the FRET experiment for c-ring rotation during ATP synthesis we cannot assign the short steps to their relative sequential position with respect to the release of ATP. We are developing a new experimental approach of monitoring c-ring rotation and simultaneous monitoring of epsilon rotation (three-color FRET with single ATP synthases). Thereby we hope to identify the timing of c-ring substeps with respect to the 120$^o$ stepping of epsilon. This new experiment should provide also the distinct dwell times of the c substeps. At the moment we cannot speculate about different dwells of c.*
*Actually we also work on the proton-driven rotation of reconstituted $F_o$ in liposomes to compare the rotary behaviour, i.e. the dwell times, in the absence of $F_1$. We aim at measuring a faster proton translocation as indicated by the work of Feniouk et al. and Franklin et al. However, these experiments are still in progress.*

p.5, middle "Effecting dwell...." should read 'Affecting .....'

*This mistyping has been changed.*

p.7,11 "...white curve ...." There is no white curve in Fig. 3.

 

*The white curve corresponding to 36$^{o}$ stepping behaviour is plotted in Fig 3 F in the revised version.*

Legend Fig.1 color coding of curves not given

*The missing color code description has been added to the figure legend.*

 