Report: Derek Nolan, School of Biochemistry and Immunology, Trinity Biomedical sciences Institute, Trinity College Dublin, Dublin 2, Ireland
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PhD Title: Bioenergetic studies in the parasitic protist *Trypanosoma brucei*: Functional characterisation of the mitochondrial F0F1-ATP synthase/ATPase complex.
Candidate: Carolina Hierro-Yap

General comments:

There is no doubt that the extensive work reported in the thesis meets the standards of effort, scholarship, rigor and novelty required for a doctoral thesis. The level of work, the range of technologies and the complexity of some of the experiments described in this thesis are exceptional and are a testament to the determination and laboratory skills of the candidate. In addition, the interpretation and analysis of the results meet the standard of scholarship required of doctoral level research. The contribution of the candidate to this field is already clear in two primary publications, including a first author paper in the Journal of Biological Chemistry, an insightful review on the trypanosomal F1F0-ATPase and an extensive set of currently unpublished results concerning the oligomerisation of the complex as well as the function of the OSCP subunit in akinetoplastic *T. brucei*.
I was also very impressed with the clarity and quality of the writing and the presentation of the data throughout the thesis. It is clear to me that a lot of effort has been put into this aspect of the work and commend the candidate and the supervisor on a job well done. It really shows.

In summary, this is an excellent PhD thesis and in my view clearly meets the standards required to recommend the awarding of a doctoral degree.

Overview of the thesis

1. Introduction: This well written introduction (25 page) covers two main topics. First, there is an introduction to trypanosomes and a description of the metabolism of the main proliferative stages: the slender bloodstream form and the procyclic form. Second, there is a good overview of the F1F0-ATPase family and the structure/mechanism/function of this electrogenic pump.
2. A clear summary of the aims of the thesis

3. A large section covering published results:
   (i) a full first author paper in JBC describing in detail the effects of the knockdown of the F1F0-ATPase in BF & PF forms.;
   (ii) A study on effect of depletion of cardiolipin in bloodstream forms, using conditional knockouts of cardiolipin synthase, published in the FASEB journal and where the candidate appears as a second author.
   (iii) A published review on the structure/function of the trypanosomal ATP synthase in Parasitology in which the candidate is a second author

4 An unpublished results section that contains chapters which

(i) Investigate the mechanism of oligomerisation of the ATP synthase. This is a structural study that combines cryo-EM of the entire ATP synthase dimer and RNAi to show that dimerization is facilitated by the conserved g-subunit.
(ii) Investigate the function of the F1F0-ATPase in an Ak *T. brucei* cell line lacking a mitochondrial genome and also the *T. b. evansi* naturally occurring Dk strain lacking the kDNA

5 A concluding remarks section, which provides a succinct summary of the extensive results contained in each section (very useful)
6 A short future persectives section which brings together the major findings and highlights future directions in which the studies may be usefully extended and also a robust justification for the use of these fascinating organisms as a system to investigate fundamental bioenergetics questions. By way of note: I agree totally with the sentiments expressed in the last paragraph!

This overview in its self clearly demonstrates the amount, the quality and the novelty of the research effort contained in this thesis. It was a pleasure to act as an external examiner and to read this thesis. On a personal level, I could help but notice the title is so similar to my own PhD of manty many years ago: "Bioenergetic studies on bloodstream and procyclic forms of trypanosome brucei".

It was a pleasure to read about work and approaches I could not have imagined back then.

My questions are a mix of philosophical and thesis specific and reflect my thoughts on the subject and reading the thesis and are really designed to discuss with the candidate her excellent work and implications of her findings.

General questions

1. I appreciate there is a quantification of the % contribution by the candidate to the published papers on page VII but could you describe briefly which experiments you performed or the date were generated you personally, to give an idea the breath of your experimental work. Could you also describe what you feel are the key findings of your work and why you feel they are important to the field?

2. From a bioenergetic point of view what do you think constitutes an energy transducing membrane such as the mitochondrial inner membrane or bacterial surface membrane, e.g. what is the key bioenergetic parameter that is common to all.

3. Why do think nature evolved a mechanism of energy coupling by ion gradients?

4. It is clear that the F1F0-ATPase is essential in both BSF and PCF, it is easier to see the requirement in PCF, generating ATP. What essential role do you think it plays in BSF where ATP levels increase when F1F0-ATPase is virtually depleted in BSF. Given that the oligomycin-sensitive ATPase activity is low in BSF (~ 30 nmol/mim.mg) compared to the rate of pyruvate production (about 230 nmol/min.mg) is ADP generation via F1F0-ATPase likely to be critical

for glycolytic activity? An interesting question here is what might be the rate of the F1F0-ATPase activity *in situ* in live BSF under fully aerobic conditions, what would most likely dictate this rate.

5 Is reversal of the F1F0-ATpase that uncommon? Increasingly we see re-wiring of metabolism and bioenergetics pathways that often involves reversal of the F1F0-ATPase, especially in the context of immune cells or cancers cells under aerobic conditions, the Warburg effect. This is similar in a way to BSF trypanosomes. Could you speculate on why cells might opt to use substrate level phosphorylation to generate ATP to generate and maintain a proton electrochemical gradient by ATP hydrolysis under aerobic conditions?

Specific questions

Introduction

The main route of carbon metabolism in BSF is glycolysis, other pathways are about 10%. Traditionally carbon metabolism in BSF cells was viewed as simply glycolysis (overwhelmingly) and a small amount of the flux going through the oxidative portion of the pentose phosphate pathway. This picture is changing could you give of this why these pathways are essential even though they function at very low activity relative to glycolysis.

What is your view on tissue specific bloodstream forms? Trypanosomes are great travellers and are found through the vasculature, interstial spaces and the CNS, exclusively extracellular they go everywhere they can but is there any real evidence for tissue specific forms , my own feeling is they are all essentially long slender or stumpy/intermediate forms.

The C subunit story is very interesting. Could you speculate on the variation in C subunits in the F0 section? What are the implications in terms proton cost per ATP? As a full rotation always produces three ATPs then the number of protons required to move through rotor per ATP produced will increase as the C-subunit number increases.

Results:

JBC paper; I would say that the effect on growth occurs secondary to the changed ADP/ATP ratio. Thermodynamically the key is how far this ratio is from the K for ATP hydrolysis.
It is not the concentration of ATP that is important, it is the ΔG for the ATP hydrolysis reaction that is key and this depends on the ATP/ADP ratio. The energy release by hydrolysis of an ATP molecule is less, not just the concentration.

Interesting that loss of a component of the F0 subunit leads to loss of F0 /stalk associated proteins (Tb2). Why might these subunits be lost but not those of the F1 portion.

Could you offer an explanation for the transient increase in the mitochondrial membrane potential in PCF following KD of Tb1?

I assume these PCF data (Fig 2 JBC paper) are 3' end RNAi on Tb1, did you ever try the 5' end given the results you got with BSF. Is there also a margin or level of knockdown where PCF could also survive or is this more critical since in PCF the F1F0-ATPase has a more central role in support ATP levels by ox phos. Would you expect the basal activity requirements to be the same?

Could you comment on the finding that AOX activity is lower Fig. 4G (JBC paper) in Tb1 RNAi cells when it looks as if the protein expression is much higher for Tb1 3' RNAi .

I would not entirely agree that higher levels of ATP could cause a decrease in glycolytic flux. They may, but what you measure directly is AOX activity in permeabilised cells, so actually the AOX activity. Better would have been to measure O_2 consumption by live cells respiring on glucose.

I have to compliment you on the Fig 4 panels C-D and Fig 6 panels B and C these are really convincing experiments.

I would agree with you that your experiments show that, as is the case for many enzymes, BSF trypanosomes do not need all the F1F0-ATPase activity they have; it seems that it only operates at about 10% of the activity for normal growth. What do you think this level implies, what is this 10% of activity essential for.....what actually drives the rate of ATP hydrolysis by this electrogenic pump? Can this level of activity really influence glycolysis?

It is interesting that in *T. brucei* accumulation of intact active F1 in Tba1 RNAi cells or TbCls conditional KD cells (along with the AAC) cannot maintain a membrane potential as is the case of *T. evansi*. The explanation are the mutations in F1 in the latter case that increase the intrinsic activity. Even allowing for a difference of rate of F1 hydrolysis it hard to see how a membrane potential would not be generated in the case of *T. brucei*.
Have you consider an alternative possibility that the mitochondrial inner membrane in these cells is more permeable to H^+ for example, the possible presence of some partial F0 without attached F1 acts as a protonophore.

TbCLs paper Figure 1 C: why not blot directly, why was immunoprecipitation employed, or is this an error.

The idea that it is the decrease in ATP levels in TbCls depleted cells is responsible for the loss of membrane potential after 24h accounts for the drop in membrane potential is interesting. Nevertheless, I am not sure it is correct, what is the effect of adding SHAM on the TMRE fluorescence.

I would also be careful about extrapolating directly the fluorescence response to the absolute value of the membrane potential; this is not a linear response. (TbCls paper Fig. 2C)....a 50-75% decrease in fluorescence is what you measure, this does not necessarily equate to a similar absolute drop in membrane potential.

TbCls paper Fig. 5B, there is a 50 drop in 0_2 consumption; did you look at pyruvate production?

What is the current view on the role of the trypanosomal specific subunits, for example the three p18 subunits.

It is very interesting and unexpected that the membrane potential is abolished by oligomycin or inhibition of the translocator in Ak cells, e.g. even *T. evansi* WT cells I am not sure I follow the explanation and how this fits with the role of "free " F1 acting as a nonspecific ATP hydrolysing activity.

Technical questions:

Western blotting can be very nonlinear at high and/or levels of the target. You generate (in JBC paper , section 1.1) very precise values for the knockdowns down to values to below 10%...e.g. 3-4% or even less Fig. 3A or Fig. 5A. Indeed sometimes, a target can barely be detected by blotting and yet a significant level of transcript can remain in the knockdown. Did you try qRT PCR to assess Tb1/PSCP transcript levels in the experiments? Often you find that when you can measure the target activity directly, say by enzyme assay, it correlates closely with the qRT transcript level but not the western blot data. For example Fig.3A it looks as if very little(none ?) of Tb1 remains after 3 days but this might be due to a nonlinear response and a threshold level of detection. See also the data (Fig. 5) in the paper on CL depletion. Here we see a 50% decrease in O2 consumption (panel B) by cells (-tet) but the AOX blots (panel C) seems to suggest an even greater loss). When you checked with qRT-PCR (figure 1) the loss of TbCls again the loss by blotting (panel c) seems greater than by qRT-PCR (panel B).
This does not change your conclusions, I think the explanation for Fig 3 and Fig 5 in terms of growth is correct, but in both cases, the level of Tb1 could be higher than you think.
Perhaps a qRT-PCR analysis would have helped here, I was curious why not.

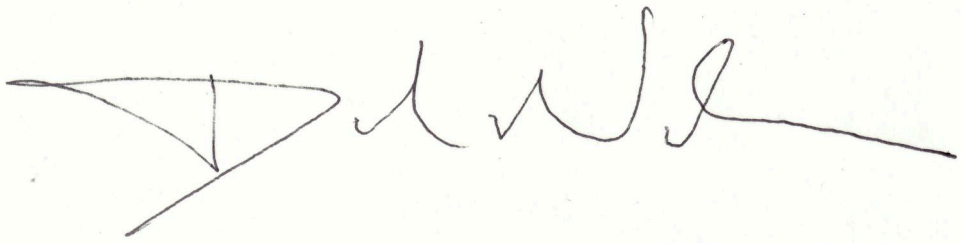
Why did you use a tagged form of Tb1 when you had a very nice antibody?

Could you explain how mitoSOX specifically measures matrix ROS

Could you explain how you measured the ATP/ADP ration, it is not so clear in the methods.
I assume the cells were lysed and PEP was used.

TbCls paper Fig. 1 panel B, am not sure I understand the units on the y-axis (pmol/(s*ml) .
Are these steady state rates of cells taken at the various times indicated?

TbCls paper Table s1; in bloodstream forms of *T. brucei* alanine aminotransferase appears to be a cytoplasmic enzyme.



Bioenergetic studies in the parasitic protist *Trypanosoma brucei*: Functional characterisation of the mitochondrial FoF1-ATP synthase/ATPase complex by Carolina Hierro-Yap

This Ph.D. thesis focuses on the functional characterization of the mitochondrial FoF1-ATP synthase/ATPase complex in *Trypanosoma brucei*. The results are divided into four topics: (a) bioenergetics consequences of mitochondrial FoF1-ATP synthase deficiency in procyclic and bloodstream forms, with emphasis on the roles of the Tb1 and OSCP subunits, (b) changes in energy metabolism of bloodstream form parasites depleted of cardiolipin, (c) role of the FoF1-ATP synthase dimerization in mitochondrial biogenesis and bioenergetics, with focus on the functional characterization of the dimer-specific subunit *g* in the insect stage of the parasite, and (d) role of subunit OSCP in the structural and functional integrity of the FoF1-ATPase in trypanosomes lacking mitochondrial DNA (akinetoplastic). The thesis is clearly written and contains a comprehensive introduction on the parasite and disease. This is a thoughtfully considered and well executed study. Overall, this thesis advances the field significantly and I have only a few comments and questions. In my opinion the thesis fulfills the requirements for successful defense.

General comments on the use of Tet-on/Tet-off system for RNAi
A number of papers have indicated that tetracyclines affect mitochondria at concentrations used typically in the control of gene expression in different eukaryotic models (mammals, worms, plants) (1-3). What do you think? Tetracyclines are also phototoxic. Were your cultures protected from light when doing RNAi?

1. Section 1.4.3 (Introduction)
Although the author indicates that in the BSF the ATP synthase functions in the reverse mode to maintain the $\Delta\Psi_m$, she does not indicate why this is important, i.e., why the BSF needs to maintain a $\Delta\Psi_m$? Why is it essential for survival of the BSF?

2. Section 1.1 (Results)
Figure 12B. Quantitative information is provided here. However, only one experiment is presented and no statistical analysis is shown. The same applies to Figures 3C and G, and Figures 5E and 5F. However, appropriate quantification of immunoblots is used in Figure 6 of Section 2.1.

Figure 2F. I am not convinced that the increase of TMRE fluorescence means hyperpolarization unless this is demonstrated by calculating the $\Delta\Psi_m$ value in mV. For example, using valinomycin and K^+ and applying the Nernst equation.

Figure 2H and 2I. Both MitoSOX and H_2DCFHDA measure reactive oxygen species in general and it is difficult to attribute detection of a particular species (superoxide anion or peroxyl radicals) to each dye.

3. Section 1.2 (Results)
Methods. $\Delta\Psi_m$ measurements using safranine O are done using a plate reader here. Was agitation maintained? Any advantage of using a plate reader?

Discussion. Two V-ATPase subunits (A: Tb927.4.1080 and E: Tb927.11.9420) are upregulated upon CL depletion. Do you have any comment on why this could happen and which is the link to the ATP synthase?

4. Section 1.3 (Results)
Nice review. Perhaps it would have been appropriate to explain here why the maintenance of a $\Delta\Psi_m$ is required for survival of BSF.

5. Section 2 (Results)
Very straightforward results. No comments

References

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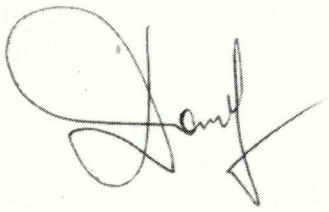

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