Academic Medical Center

University of Amsterdam

Amsterdam, 18-05-2006

Dear Julius and Zdeněk,

After having read the proposed work of Zdeněk Verner, student in the Laboratory of molecular biology of protists in České Budějovice, I can happily state that, according to me, the work is more than acceptable to grant him a Masters degree.
I congratulate you both with the amount (the sheer number of different techniques used is really impressive) and quality of the work.

Having stated this, however, I must stress that I still have some remarks and questions regarding the content of the Master thesis. I will focus on scientific considerations here (the slight language errors in the manuscript mostly stem from difficulties with the correct use of pronouns that eastern europeans -understandibly- are plagued with*).

1. In the introduction (page 2) a.o. the *T. brucei* kDNA is described as particulars of this model organism are given. The maxicircle is described as being approx. 40 kB in size. Actually, it is 23 kB. Presumably the introduction switched to trypanosomatids in general (and 40 kB is a good general value in that case) but this is not made clear.

2. On page 9/10 the consequences of the 'knock downs' of the 3 putative complex I subunits (**ndhK**, **ndh40** and **ndh50**) are given:
- No growth effects of the individual RNAi's
- No effects on mitochondrial membrane potential
- No effect on overall NADH dehydrogenase activity (which, by the way, is completely rotenon insensitive!).
To still want to draw conclusions from the 'knock downs' regarding complex I functioning in trypanosomatids seems to me to be rather hazardous.

Regarding 'Downregulation of the nuclear-encoded subunits of the complexes III and IV disrupts their respective complexes but not complex I in procyclic *Trypanosoma brucei*' by Anton Horváth, Eva Horáková, Petra Dunajíková, Zdeněk Verner, Eli ka Pravdová, Iveta lapetová, L'udmila Cuninková and Julius Lukeš, I have the following remarks.

As Zdeněk states there has been a long controversy about the existence of complex I in procyclics. The existence of the **genuine** complex I in **procyclics** is said to be demonstrated in this article. I must say that I am **STILL** not completely convinced by the arguments and experimental findings.
To makes sure that the discussion will not be semantic in nature:
- the presence of mt. (occasionally extensively edited) and nuclearly encoded transcripts for 'complex I - like' subunits plus

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- the claimed rotenon sensitive NADH dehydrogenase activity in stumpy blood stream form trypanosomes (Bienen E.J. *et al* Mol. Biochem. Parasitol. **45**, 185-192) make it clear that a 'complex I - like' mt inner membrane complex is sometimes present in trypanosomes.
But the existence of mitochondrial genes and nuclearly encoded subunits for NADH dehydrogenase as such does not prove they are used in procyclics to form a classical complex I as implied on page 2 of the article. They could be used only in the short stumpy bloodstream forms and this would explain their presence in the respective genomes.

The question than becomes: is what you're looking at the bona fide complex I?

The arguments against this are:

1. Most of the observed editing patterns do not seem to correlate with the expression of complex I in procyclics.

2. In *C. fasciculata* (Speijer *et. al.* (1997) Characterization of the respiratory chain from cultured *Crithidia fasciculata*. Mol. Biochem. Parasitol. **85**, 171-186.) we fond no trace of complex I while we easily demonstrated the presence of *all* the other respiratory chain complexes. This highly cultured strain could be 'unnatural', but at least demonstrates respiration starting with complex II instead of complex I in 'insect forms' (although this of course is NOT *T.brucei*).

3. It is stated categorically that the histochemical staining in Figure 6B of the article at about 650 kD must be that of complex I and can not be the result of an alternative NADH dehydrogenase activity. Its relative small size makes this less likely (normally complex I is the by far the largest respiratory chain complex), especially since multimerization is the rule in migration on BNE gels (Speijer *et. al.* Mol. Biochem. Parasitol. **85**, 171-186, again; sorry about that).

4. We do not see anything resembling a complex with even a few different subunits in between complex IV and III (or to the right of both of them) in figure 5 of the article, as predicted by the histochemical staining in Figure 6B of the article. The 2D shown in figure 11 of the thesis (I know this is a difficult technique to get to work optimally!) is not really convincing when it comes to a complex of subunits associated with the activity in the first (native) dimension. The western blot that goes with it, at least shows that it is POSSIBLE that the ndh50 protein forms part of the complex responsible for the NADH dehydrogenase activity in the first dimension but as all these activities are rotenon insensitive this could still be called an alternative NADH dehydrogenase complex.

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Concluding: are you looking at the bona fide complex I? I really think we can not tell yet. But at least this constitues a gallant effort to answer these difficult questions and I congratulate you both in advance with Zdeněk's Masters degree.

With kind regards,

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* Do not take this the wrong way: the Dutch, for instance, have terrible accents. I envisage Hell as populated by Dutchmen who have to communicate in English (myself included).

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19 May, 2006

Review of the Master Thesis "Functional study of three putative subunits (ndhK, ndh40 and ndh50) of complex I (NADH:ubiquinone reductase) in procyclic form of *Trypanosoma brucei*" by Zdeněk Verner, BSc.

Summary

The goal of this thesis was to obtain evidence for the expression of mitochondrial NADH:ubiquinone oxidoreductase (also known as complex I) in the procyclic (insect stage) of the parasite *Trypanosoma brucei*. The existence of this complex in *T. brucei* is controversial and to provide definitive evidence for or against its expression is of great importance for the field. In a second project, Zdeněk Verner participated in the functional analysis of nuclearly encoded subunits of respiratory complexes III and IV in *T. brucei*.

Zdeněk Verner pursued two complimentary approaches. First, using a reverse genetics approach, he silenced expression of three putative subunits by inducible RNA interference (RNAi) and analyzed the effects on cell growth and mitochondrial NADH dehydrogenase activity. Surprisingly, although gene expression as measured by Northern analysis was significantly knocked down in all cases, no significant effect on mitochondrial NADH dehydrogenase activity was observed. Cell growth was also unaffected. In the second approach, Zdeněk Verner provided one of the putative subunits with an epitope tag, expressed the construct ectopically in *T. brucei*, and asked whether the protein would co-purify with NADH dehydrogenase activity in fractionated mitochondrial lysates. Indeed, analysis by two-dimensional gel electrophoresis showed that abundance of the tagged protein peaked together with NADH dehydrogenase activity.

In general, the approaches are sound, the experiments are carefully carried out and mostly well controlled, the data are of high quality, and the conclusions are valid. A more detailed introduction to complex I (what is known about its structure, the function of its subunits, and its inhibitors?) would have been helpful and some of the experiments would have benefited from additional controls and/or reproductions. It would have been informative to discuss in more detail the findings of the present study in the context of published data.

Overall, this is a good and interesting study. The data on respiratory complex I, together with the published study on complexes III and IV, will provide the field with important new insights into this still poorly understood aspect of trypanosomatid biology.

Detailed comments on individual sections

1) Introduction. This section offers a concise, up-to-date and informative introduction to energy metabolism and mitochondrial function in *T. brucei* and sets the stage for the problem investigated (one correction: the maxicircle size in *T. brucei* is ~23 kb, not 40 kb). However, it would have been helpful to include a more detailed description of complex I: What is known about its structure? (The 3-dimensional structure of the hydrophilic domain of the *Thermus thermophilus* complex was published earlier this year). What is known about the functions of its individual subunits? What inhibitors are known and how do they act? Why were subunits ndhK, ndh40 and ndh50 chosen as targets?

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2) Material and methods. This section provides the right level of detail, enabling the reader to understand the experimental procedures. One suggestion: the source of the pLew79-MH-TAP plasmid (lab of Marilyn Parsons) should be included.

3) Results

3.1) Computational analysis of putative complex I subunits. This is a thorough analysis that convincingly demonstrates that genes Tb09.244.2620 and Tb927.5.450 encode orthologs of subunits ndh40 and ndh50, respectively. A reciprocal blast analysis could have been used to provide additional evidence that the identified genes truly represent the closest homologs of ndh40 and ndh50 in other organisms, and *vice versa*.

3.2) Generation of knock down cell lines and there phenotypic analysis. The growth curves and Northern blots are of good quality and demonstrate efficient (but not complete) knock down of target gene expression. I assume the bands pointed at by the arrows correspond in size to what would be expected for the respective mRNA species? The inclusion of size markers would have been helpful in evaluating this. Given the lack of an effect on NADH dehydrogenase activity (see below), it is not surprising that the mitochondrial membrane potential ($\Delta\Psi m$) was unaffected. The observation that $\Delta\Psi m$ decreases at higher cell densities is interesting, however, and could be investigated in more detail in the future.

NADH dehydrogenase activity with Q2 and ferricyanide as acceptors was tested and no decrease upon silencing of either subunit was observed. The effect of the inhibitors rotenone (considered specific for complex I) and DPI (general inhibitor of flavoproteins) was also tested. Only DPI showed significant and consistent inhibition. Measurements with combined silencing and chemical inhibition were carried out only once for each combination and are therefore difficult to interpret. These experiments should have been either repeated or omitted from this study.

Finally, the effect of the knock down on histochemical staining of NADH dehydrogenase activity in blue native gels was evaluated. Again, no effect was observed, but DPI at 0.4 mM completely abolished staining. This concentration is very high compared to studies published for other organisms. Were lower concentrations tested as well? Was the effect of rotenone or piericidin A (two more specific inhibitors) tested?

3.3) Ectopic expression of epitope-tagged ndh50. The Western analysis shown in Fig. 10 demonstrates that the tagged protein is expressed and targeted to the mitochondrion, although the lack of control fractions and antibodies makes it impossible for the reviewer to evaluate this claim. Mitochondrial fractions were solubilized and fractionated by 2D gel electrophoresis. This experiment provides some evidence that the tagged subunit is indeed incorporated into the putative complex I, since the peak of abundance of the tagged proteins correlates with NAH dehydrogenase activity. This is an important experiment with an interesting result that provides a good basis for further studies.

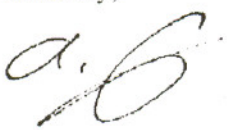
3.4. Summary. I agree with the conclusions presented in this section, except the first one. Presence of a stable mRNA is not proof of expression of the gene. Additional evidence, e.g. with the help of specific antibodies or proteomics methods is needed to substantiate this claim.

4) Discussion. This section provides a good discussion of the observed results, valid conclusions are drawn, and good suggestions for follow-up studies are made. The discussion could have been benefited from a more thorough discussion of the findings presented here in the context of data from the literature. For instance, how can the lack of rotenone-sensitivity of NADH dehydrogenase activity observed here be reconciled with the rotenone-sensitivity reported by the Beattie lab? What could be the explanation for the lack of an effect of silencing of either subunit - in particular the FMN/NADH-binding subunit ndh50 - on the histochemical staining? Does that mean that the knock down was not efficient enough? Or could this be evidence that the observed staining is not due to complex I activity? In this context, it might have been mentioned that the Beattie lab also observed a single band upon staining of blue native gels with NADH/NTB and attributed this staining to a type II NADH dehydrogenase (Fang & Beattie, 2002). As suggested in the thesis, the generation of specific antibodies will be helpful to address these questions. The generation of true null mutants for selected

putative complex I subunits or silencing of candidate genes encoding alternative NADH dehydrogenases should also be considered. The generated cell line expressing TAP- and epitope-tagged ndh50 should also provide a very valuable tool to investigate the identity of the stained complex.

I hope these comments are helpful.

Sincerely,



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