Functional study of the SUF pathway in the cell of *Monocercomonoides exilis* and *Paratrimastix pyriformis* by Bc. Marie Zelená

REVIEW

Once again, I was asked to assess a diploma thesis in English. The last one was a pure linguistic hell and I kept wondering as why the poor soul decided not to use her native language and set on a path of a reviewer's torment. The thesis of Ms. Zelena has been on the exact opposite side of a spectrum. The language? BEAUTIFUL. References? Almost perfect. Literature review? EXCELLENT.

The thesis itself is focused to five aims (to prepare a recombinant version of MeSufC; to localize the Suf pathway in *M. exilis* and *P. pyriformis* using IFA; to develop an assay for measurement of SufC; to co-express SufB/C and to characterize their interaction in vitro) and is truly a pleasure to read. With this in mind, I have several minor notes on the following parts.

The INTRODUCTION gives a clear picture about the two organisms and FeS cluster assembly pathways. Formally, there are two missing references (1] Fig. 1 Karnkowska and Hampl, 2017; 2] Fig. 10 Goldsmith-Fischman et al., 2004); since both come from figures, I believe it is a problem of reference manager not being able to work with picture captions. The other formal note points to a positioning of sub-headlines: as SufA is not part of a scaffold complex (2.3.5.2), I would put it as headline 2.3.5.3 instead of 2.3.5.2.4. Next, when speaking about SufB, the reader is pointed towards figure 10 (page 11), however, Fig. 10 speaks about SufE; I believe the intentions are towards figure 12. Speaking about figures, I did not find any clue as what actually is present on Fig. 1C; neither caption nor text refers to that. Also, Fig. 6 depicts ISC pathway to be NADP-dependent, while in the text you describe it as NAD-dependent. Lastly, the organelle of *Mastigamoeba balamuthi* is not classified as mitosome (page 17: "In the case of *M. balamuthi*, dual localization of the pathway in the mitosome, as well as the cytosol, has been confirmed experimentally (Nývltová et al., 2013)."); the authors themselves call it „…an intermediate stage between "classic" mitochondria and the reduced mitosomes of *E. histolytica…"*

Regarding the factual part, I have following questions:

1) Your description of FeS clusters is focused on 2Fe2S and 4Fe4S clusters. What is the ratio of other classes built by prokaryotic and eukaryotic cell?

2) I did not find any information regarding classes of FeS clusters built by CIA pathway. Could you comment on that?
3) On page 17, you speak about LGT as a means to introduce Archeic genes for Suf pathway into a eukaryotic cell dwelling in the same environment as the Archea. Then you go on with the sentence: "However, in the case of the human gut parasite *Blastocystis hominis*, this event is hard to imagine, even though this parasite does produce cysts which are released to the outside environment…" Would it be possible to speculate about environment of ancestors of *B. hominis*? Where did they live before raise of hominids? Where did they live before raise of multicellular organisms?

Next chapter, MATERIALS AND METHODS is written clearly and covers everything from cultivation through gene amplification and protein expression and purification to techniques of enzymology. There is just a minor discrepancy regarding unit conversion in formula at page 33 (extinction coefficient is given in mols while the resulting concentration is considered in mmols). Thus, the following questions are rather of a philosophical nature as I do not consider myself being strong enough in general enzymology:

1) You decided to optimize conditions for measurements of the ATPase activity using a coupled reaction (ATPase-pyruvate kinase-lactate dehydrogenase). You manipulated pH, ionic strength and metal cofactors' concentration. Could you provide a solid evidence that ATP and the coupling enzymes are stable with constant activity under the tested conditions?
2) Moreover, as experience with NADH:ubiquinone oxidoreductase activity taught me, the activity might be tremendously different with respect to the buffering system at the same pH: using Tris-HCl gives no activity while NaPi works like a charm at the same pH 7.4; hence what is your justification for using MES/HEPES/Tris buffers over any other combination?

To me, the RESULTS section appears to be the weakest part of thesis. Not that the results per se are weak but I missed a lot of supporting information. I was not able to find a single information about the sequences of the expressed protein; hence, I am left with expressions "approximate Mw" (pg. 37, last sentence; pg. 38, Fig. 24) and "expected Mw" (pg. 39, Tab. 33). Dilution of antibodies? Not mentioned except for "…(dilution ratio depending on the antibody used)…" (Materials and Methods, 4.4, pg. 29). For immnunofluorescence, the reader is left without a clue whether widefield or confocal mode was used. Figures 25-27: Am

I looking at deconvolved pictures? Maximum intensity projection or a single slice? No clue. However, my curiosity was caught by the co-expression of SufB/C:

1) SufC-HA seems to undergo rather massive degradation (Fig. 33). Did you try swapping the tags as both SufC- (Fig. 23) and SufB-His (Fig. 33) show a nice single band?
2) Upon size-exclusion chromatography, the fractions were separated on SDS-PAGE and stained using Coomasie brilliant blue. During discussion (pg. 52, the third line and on), you state "…no bands were detected in fraction 10 … This suggests that SufB may be capable of forming very large complexes with itself, yet its amount was so low it was barely detected on the gel, …". To me, this statement begs you to perform more sensitive staining method. Why did you use this instead of Silver staining?

Finally, the DISCUSSION is again very well written. One after another, the results are commented and put into a context. Reading it, the following apparent questions and comments popped in my mind:

1) The third paragraph on page 48 describes western blots and subsequent immunoprecipitation. Have you considered any simple fractionation of the cell to relatively increase the abundance of your target protein and possibly get rid of the cross-contaminating actin?
2) The first paragraph on page 50 discusses co-expression of SufB/C. You state: "However, when trying to measure the ATPase activity of the whole protein mixture, the mixture still displayed ATPase activity, proposing that some SufC has conserved its activity regardless of its binding to the scaffold protein." Can you rule out that the activity stems from a contaminating bacterial ATPase(s)?
3) Lastly, I was surprised that at least some of the FPLC fractions were not analysed by MS.

Overall, the diploma thesis is of EXCELLENT QUALITY and I strongly recommend it for defence.

RNDr. Zdeněk Verner, Ph. D.