UNIVERSITY OF COPENHAGEN
DEPARTMENT OF FOOD SCIENCE (FOOD)

Tomas Dolezal
Associated professor and head of the Department of Molecular Biology and Genetics
Faculty of Science, University of South Bohemia
Street: Branisovska 31c
City: Ceske Budejovice, ZIP: 37005
Czech Republic

Evaluation of the PhD thesis by Marek Pazderník

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SECTION FOR INGREDIENT AND DAIRY TECHNOLOGY (IDT)

ROLIGHEDSVEJ 26, DK-1958
FREDERIKSBERG

TEL 45 61344637
MOB 45 61344637

peje@food.ku.dk
http://plen.ku.dk/english/employees/?pure=en/persons/310667

REF: PEJE

Marek Pazderník has submitted a thesis entitled "Light-harvesting like domain of the cyanobacterial ferrochelatase". The topic of the thesis is characterization of the functional, molecular and physiological roles of a domain of the cyanobacterial ferrochelatase (FeCh) which resembles a part of the light-harvesting proteins (LHC) found in higher plants and which decent from smaller membrane bound proteins that can bind chlorophyll and carotenoid pigments. The presented research shows that the cyanobacterium Synechocystis actively maintains a certain ratio between Chl and heme. A balance that is shifted towards heme during stress conditions such as low temperature combined with high-light. In a mutant possessing an aberrant FeCh, lacking the Chl-binding and dimerization motif, this stress-induced heme synthesis is not observed suggesting that the FeCh LHC-motif facilitates channelling of the common ferrochelatse and Mg-chelatase substrate protoporphyrin IX (PPIX) towards heme. Interestingly, the data show that the FeCh LHC-helix localizes the FeCh enzyme to a CurT dependent membrane domain. CurT has a role in shaping special domains of the Synechocystis thylakoids where assembly of PSII seems to take place. In the thesis it is also shown that the FeCh LHC-helix dimerizes and binds Chl when the FeCh activity is lowered supporting a role of the LHC-helix in balancing the synthesis of Chl and heme. On the basis of the findings Marek Pazderník propose that the role of the FeCh LHC-domain is to monitor the availability of Chl during the assembly of photosystems and coordinate Chl availability with the synthesis of heme.

The thesis is structured in an Introduction leading to the Objectives and followed by a Methods chapter and a combined Result and discussion chapter. The thesis is concluded with a thorough General discussion and a concise conclusion. In addition there is an Appendix. Included is also a PDF of a paper published in Journal of Biological Chemistry (JBC) where Marek

Pazderník is first author. The published paper reports part of the data presented in the Result and discussion chapter. JBC is a leading journal in biological chemistry with an impact factor of 4.1.

The Introduction generally gives a good overview of the research field of regulation in biological systems (DNA, proteins and other biomolecules). Photosynthesis is also introduced and a futile speculation on making humans photosynthetic is presented and used to motivate why we should focus research interests in photosynthetic organisms. The argumentation is highly speculative and unfortunately reveals a lack of basic bioenergetics understanding. As idea it is provocative but not really suited for a PhD introduction. However, this is only a minor objection as the reminder of the Introduction instead focusses on biosynthesis of chlorophyll and heme, Chl-binding proteins and gradually focusing on the Light-harvesting complex like proteins and the relevance for the LHC domain of the FeCh. Thus, the Introduction nicely gives the state of the art of the field and prepares the reader for the reminder of the PhD thesis. A few well-illustrated figures are presented in the Introduction which facilitates reading of the Introduction. Thus, in general the Introduction makes up a good, well-structured and qualified primer for the reminder of the thesis.

The Introduction ends with the Objectives of the thesis which are clear and concise.

The Methods chapter is generally well-written and informative, however, in some parts minor details are missing:

- Choice and use of Synechocystis strain – glucose tolerant and WT-P substrain.
- Details of quality assment of isolated RNA and DNA.
- Transformation using chromosomal DNA (page 16).
- Consecutive promoter?
- microM or micrometer?
- Methods for breaking cells – what is the difference between the methods used and how is the efficiency assessed?
- Methods for solubilization before chromatography is not clear from the thesis although it is clearer when reading the JBC paper.

The Results and discussion chapter reveals a wealth of data and information. It has a logic progression answering one hypothesis and leading to the next. The experiments presented are of high quality and the results obtained indeed progress our understanding of the FeCh in general and in particular the role of the C-terminal LHC-domain of the protein/enzyme molecule. The results obtained are interpreted in context of the current literature and

demonstrate overview and scientific maturity of the student. Some of the data are already published in the enclosed JBC paper but there are data for at least another high-quality publication.
In the following I will high-light some points that could have been improved and that can be discussed at the defense:

- The 10-line preface in 4.1 is a bit confusing and it is not clear what the purpose is and what the hypothesis is.
- The conclusion that activity of FeCh is likely inversely reflected in the rate of the de-novo Chl synthesis is solely inferred from an assumption as FeCh activity is not measured directly. Also in Fig. 6b, a ratio is given but it is not clear whether Chl RAD or Chl Abs changes. The actual values would have been helpful.
- In Fig. 7, is the loading based on number of cells?
- The demonstration that f.FeCh binds pigments (Fig. 9) is really nice. How do you estimate the stoichiometry of the pigments bound per protein?
- Fig. 12 and elsewhere – "minute fractions" – I am not sure this is what you mean.
- Is it safe to assume that the extinction coefficient and fluorescence constant are the same for both Chl populations? (page 33).
- In Fig. 18: how do you envisage the binding of the two carotenoids?
- The proposed interaction of FeCh with CurT is really interesting and will have significant consequences for our understanding of FeCh, CurT, membrane curvature and assembly of pigment-protein complexes in both cyanobacteria and plants.
- The analysis of the cross-linked products with FeCh seems thorough and detailed giving strong confidence in the data.
- Section 4.3 addresses the physiological role of the FeCh LHC motif is a major contribution and the first time this has been performed systematically. The physiological role is subtle (lower temperature combined with higher light intensity) but appears sound and indeed real.
- For the transcriptomic analysis (4.3.4) 2 biological replicates are used. Is this giving enough statistical power to make safe conclusions?
- Is PsbA2 the same as psbAII?
- The proposal that differences in carotenoid and heme levels and retarded growth of the mutant are likely caused by translational or post-translational defects requires a bit more explanation.

As partial conclusion, the Result and discussion chapter reveal a large number of well-planned and well-executed experiments and a mature interpretation of the data demonstrating the scientific abilities and qualities of the student.
This if further substantiated in the General discussion. In this chapter all the data obtained is discussed in a bigger picture. The function of the FeCh LHC domain is discussed and different possibilities are assessed. A

mechanism of the dimerization is proposed in Fig. 38 and pigment binding and enzyme activity modulation is discussed. Interesting novel functions of the FeCh in localization and involvement in PSII assembly are proposed. I think some of this is novel and original and is direct outcome of the experimental work performed and presented in this thesis.

The large amount of systematic and well-performed experimental work presented by Marek Pazderník in this thesis and the fact that the findings have progressed our understanding of the molecular role and functions of the LHC domain of the FeCh enzyme as wells as the physiological role of the FeCh certainly warrant a defence of this thesis. I therefore warmly recommend this thesis for defence.

Yours sincerely,

Poul Erik Jensen

Poul Erik Jensen
Professor in Plant Based Food Biochemistry

UNIVERSITY OF LIVERPOOL

Dr Daniel Canniffe

Institute of Integrative Biology
University of Liverpool
Biosciences Building
Liverpool
L69 7ZB

T 0151 795 7733
E d.canniffe@liverpool.ac.uk

http://www.liv.ac.uk
http://www.liv.ac.uk/integrative-biology/

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Dear Head of Ph.D. Committee,

Please find below my review of the doctoral thesis of Mgr. Marek Pazderník, entitled "Light-harvesting like domain of the cyanobacterial ferrochelatase".

Ferrochelatase (FeCH) is an enzyme that catalyses a reaction essential for life on Earth, namely the insertion of ferrous iron (Fe^{2+}) into the macrocycle of the tetrapyrrole protoporphyrin IX, yielding haem B (protohaem), a cofactor involved in energy transduction and redox reactions in almost all studied organisms. The FeCH found in cyanobacteria contains a C-terminal extension predicted to act as a chlorophyll-binding domain; this extension shares a consensus sequence found in chlorophyll-containing antenna proteins of photosystems I and II in all oxygenic phototrophs. The C-terminal extension is conserved in the FeCH found in the chloroplasts of eukaryotic phototrophs, consistent with the plastid progenitor being a cyanobacterium. The thesis of Marek Pazderník examines the role of this FeCH extension in both native and plant enzymes in the model cyanobacterium *Synechocystis* sp. PCC 6803.

The introduction to the thesis is an unconventional and entertaining read; I particularly enjoyed the perhaps tongue-in-cheek suggestion of the development of symbiotic human skin cells containing cyanobacteria, used to provide organic carbon to supplement the human diet and alleviate our dependence on crops. The sea slug *Elysia chlorotica* supplements its diet in a similar way, maintaining the chloroplasts of the alga it feeds on for many months, becoming a 'phototrophic mollusc'. The section ends by mentioning the attempted introduction of photosynthesis into heterotrophic organisms, this could be updated by citing the very recent, complementary papers detailing the autotrophic conversion of heterotrophs *E. coli* and *Pichia pastoris*.

In the experimental section of the thesis the candidate demonstrates that the C-terminal extension of FeCH is able to bind chlorophyll *a* and carotenoids *in vivo* under conditions in which free chlorophyll accumulates, in this case with the addition of an inhibitor of FeCH. Under these conditions, FeCH can be purified as a dimer, rather than a monomer, as is the case when free chlorophyll does not accumulate. The fluorescence from the bound chlorophylls of dimeric FeCH is quenched to a large degree, potentially by the bound carotenoids, suggesting that a photoprotective mechanism invoked by the availability of phototoxic free chlorophyll, sensed by the FeCH enzyme, has evolved. It would be of interest to know if dimeric FeCH can be isolated if

chlorophyll accumulates via a mechanism other than direct FeCH inhibition (perhaps by supplementation with glucose/ALA/MgP etc.)?

The work goes on to describe interactions between FeCH, mediated by the C-terminal extension, with other proteins, revealed by switching from thylakoid solubilisation with β-DDM to digitonin, initially indicating an interaction(s) with larger molecular weight complex(es) via migration on native gels, that is reversed when the C-terminus is missing. Interaction partners were identified by peptide mass spectrometry, revealing a strong interaction with CurT, a protein involved in membrane curvature, and the robustness of the interaction was reinforced via chemical crosslinking, indicating that the linker region between the catalytic region of FeCH and the C-terminal domain is involved in this CurT interaction at the cytoplasmic surface of the membrane. The interaction with CurT is hypothesised to locate FeCH at sites of photosystem II biogenesis. Confocal localisation of FeCH in whole cells was unfortunately inconclusive due to weak emission from the fused fluorescent protein, although non-uniform signal was observed – it would be interesting to know if a uniform, albeit weakly resolved signal, would be seen in a citrine-fused FeCH lacking the CurT-interacting residues?

Lastly, the physiological role of FeCH's C-terminal extension was studied, indicating that a previously-observed sensitivity to high light of a strain lacking the C-terminus was not replicated in a more genetically stable background. Rather, a phenotype was observed under both cold and high light stress. This phenotype was also initially observed when two charged and conserved chlorophyll-binding residues in the extension were replaced with non-polar amino acids, although this strain acclimated to the conditions over time. The chlorophyll-binding mutations also prevented FeCH dimerization upon chlorophyll accumulation - it would be interesting to know if single Glu or Asn mutations displayed the same phenotype? Transcriptomic analysis provides no clear explanation for the stress response of the mutant lacking the extension, although interestingly it displayed significantly higher expression of *psbAII* under standard growth conditions while displaying no growth phenotype, indicating that turnover of photosystem II D1 is even greater than in the WT. These data go against the suggestion on page 56/57 that "accumulation of heme-binding protein(s) was severely impaired in the mutant", or at least is not borne out at the RNA level. Perhaps probing with haemoprotein antibodies or proteomics would be better approaches to resolve the response in the future.

The candidate has published a first-author paper in a greatly-respected, peer-reviewed journal, and so has satisfied this requirement of his Ph.D. programme. The thesis is clear, well-written and concise, demonstrating a flair for engaging, scientific writing and a meticulous experimental approach, standing the candidate in good stead for a promising scientific career. I therefore strongly recommend Marek Pazderník be awarded a Ph.D. degree, and look forward to a lively discussion at his defence.

Yours sincerely,

D. P. Calle D. P. Calle

Daniel Canniffe