University of South Bohemia in České Budějovice

Faculty of Science

Ph.D. Thesis

Evolution of the tetrapyrrole synthesis in eukaryotes

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Annotation:

This thesis focuses on the nature of heme metabolism in various eukaryotes. One of the aims was the elucidation of the origin of the unique heme biosynthesis pathway in apicomplexan parasites through a comparative study of their photosynthetic relative *Chromera velia* combining molecular biology, biochemistry and bioinformatics approach. Using similar strategy, I have also investigated the origin and spatial organization of tetrapyrrole biosynthesis in *Euglena gracilis*. Based on the phylogenetic data I described the complex evolution of heme metabolism in kinetoplastid flagellates including pathogenic trypanosomes. I revealed that one of them (*Phytomonas*) does not require heme for viability by the combination of various biochemical and molecular biology experiments and bioinformatic analyses.

Declaration (in Czech):

Prohlašuji, že jsem disertační práci vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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V Českých Budějovicích, 27. září 2011 .....

Luděk Kořený

The research group leaders, who are also corresponding authors in the manuscripts included in this thesis, hereby confirm that Luděk Kořený contributed the principal part to all these publications. He conducted most of the experiments, analyzed the data and co-wrote the manuscripts.

Ing. Miroslav Oborník, Ph.D.

Prof. RNDr. Julius Lukeš, CSc.

Acknowledgements:

I am sincerely and heartily grateful to both my supervisors, Miroslav Oborník and Julius Lukeš, for allowing me to work on such an exciting topic. I very much appreciate their excellent guidance, support and patience and I thank them and all the other lab members for creating such a great and inspiring atmosphere for work.
I am truly indebted and thankful to Silvie Fexová for her constant support, bright ideas and critical reading of almost everything I have ever written.

Overview

During postgraduate studies I mainly focused on the nature of heme metabolism in various eukaryotes. To date, the studies of heme biosynthesis have been confined to just a handful of model organisms, which mostly belong to metazoans and higher plants and thus represent only a fraction of extant eukaryotic diversity. Importantly, this biosynthetic pathway is responsible for the production of heme, as well as precursors for the synthesis of chlorophyll, which qualifies it as one of the most crucial biochemical processes in virtually all eukaryotic cells, and is thought to be absolutely indispensable for survival of great majority of organisms. However, as my research reveals, there are intriguing differences in the spatial organization and evolutionary origins of the pathway components among various taxa. These features have made the heme biosynthesis an unexpectedly promising candidate for drug targeting.

My postgraduate research produced important insights in the evolution of heme biosynthesis, with profound biological implications. The key findings relate to the elucidation of the evolution of heme metabolism in the main parasitic lineages. I and my colleagues revealed the origin of the unique heme biosynthesis pathway in apicomplexan parasites, including the causative agent of malaria, which spans three cellular compartments and its origin was so far a complete puzzle. We managed to decipher this problem through a comparative study of the free-living photosynthetic relative of Apicomplexa, the recently discovered alga *Chromera velia* (Moore et al. 2008. Nature 451:959-63). To understand the tetrapyrrole biosynthesis of this interesting organism, I searched its presently available 454 genomic reads for genes of heme biosynthesis. I used the obtained sequences to design specific primers, amplified cDNA sequences by RACE and then performed phylogenetic analyses and protein localization predictions. Results of these analyses shed light on how the pathway operates in *C. velia* and allowed us to draw novel conclusions about its evolution into the unique heme synthesis pathway known in the Apicomplexa. Interestingly, our data provide evidence that in contrast to other phototrophs, in *C. velia* the first step of both heme and chlorophyll synthesis takes place in the mitochondria using the specific mitochondrially-formed precursors glycine and succinyl-CoA, which is how the pathway starts in heterotrophs. On the other hand, subsequent steps of the pathway are located in the plastid and carried out by enzymes of plastid origin and in this respect *Chromera* resembles other photosynthetic algae. It thus seems that the final steps of heme biosynthesis were re-located

from the plastid to the cytosol and mitochondrion after the apicomplexan protists abandoned photosynthesis and became parasites. This corresponds to our hypothesis that it is the trophic mode, which defines the place where the products of the pathway are synthesized. The possibility that *Chromera* produces both heme and chlorophyll from the common mitochondrially-derived precursors as suggested by the obtained genomic data, would make it the only known organism employing such a mechanism. Therefore, we decided to test this hypothesis by biochemical experiments using C14-labeled precursors and their *in vivo* conversion into chlorophyll. TLC analysis of the chlorophyll extracted from these cells revealed that *C. velia* is able to synthesize chlorophyll from glycine but not from glutamate (the precursor used by all phototrophs), confirming our hypothesis. These important findings were recently accepted for publication in the journal *Plant Cell* (with me as the first author).

I employed similar approach also in the project aiming to elucidate the origin and spatial organization of tetrapyrrole biosynthesis in another protist - *Euglena gracilis*. The results of phylogenetic analyses and *in silico* localization predictions using the available cDNA sequences clearly showed that this secondary alga possesses two independent pathways that have distinct origins and serve different cellular compartments. One pathway comes from the heterotrophic host of the secondary endosymbiosis (exosymbiont) and resembles the heme synthesis of heterotrophic eukaryotes, while the other one is mostly derived from the endosymbiotic alga and produces chlorophyll and heme for the plastid. These findings have already been published (Kořený and Oborník 2011. *Genome Biol. Evol*. 3:359-64).

I also investigated the heme metabolism in other unicellular eukaryotic parasites, namely the trypanosomatids, which are known to be heme-auxotrophs. One of the objectives of my PhD project was the evolutionary reconstruction of the loss of the pathway in this group that includes important human parasites such as the sleeping sickness-causing *Trypanosoma brucei*. Based in part on my phylogenetic data, I and my colleagues were able to formulate new and interesting hypothesis on the evolution and loss of heme synthesis in this group. We postulate that the heme synthesis had been lost already in the ancestor of trypanosomatids and was then partially or completely rescued by the acquisition of individual bacterial genes or bacterial endosymbionts (Kořený, Lukeš and Oborník 2010. *Int. J. Parasitol*. 40:149-56).

The most striking finding I made during my PhD was that *Phytomonas serpens*, a trypanosomatid parasite infecting plants, is able to survive without any heme. I was able to grow this flagellate in a chemically defined medium without heme and consequently, no heme was detected in the cell extracts using a very sensitive HPLC assay. The fact that this

organism is unable to synthesize heme of its own was further confirmed by the lack of heme biosynthesis enzymes in the draft of its genome. However, when heme was added in the medium, its fraction was detected in cell extracts suggesting that *Phytomonas* did not lose the capacity to use it for some functions when available.

In order to find out how *Phytomonas* can survive without the key heme-dependent activities and possibly identify functions still using heme if available, I and my colleagues decided to dissect cellular processes, in which heme was so far considered to be universally indispensable. Indeed, we have experimentally proven that in this kinetoplastid flagellate heme plays no role in electron transport in the respiratory chain, protection against oxidative stress and desaturation of fatty acids. When available, heme is used for the demethylation of lanosterol in the ergosterol biosynthesis pathway, but *Phytomonas* is equally viable without this activity, which is so far unprecedented, making it the only known eukaryote able to survive without heme. These findings were revealed by a combination of various bioinformatic, biochemical, molecular biology and enzymology methods including analyses of fatty acids and sterol composition by gas chromatography and TLC analysis, oxidative stress assays and comparative genomics. Moreover, respiratory complex II was detected in native gel by histochemical activity staining and specific antibodies, and its enzymatic activity was measured depending on the presence or absence of heme. These findings are prepared for submission to one of the top scientific journals, with me as the first author.

All in all, I have spent the last five years studying heme metabolism in various eukaryotic lineages and I feel that this work gave me sufficient background and insight to summarize the current state of this rapidly evolving and exciting field in a review that follows and which is also intended to serve as an introduction to this subject. This review not only builds on previous knowledge including my own work but also brings new information gained from recently finished genome projects and is intended for publication.

Review

EVOLUTION OF HEME METABOLISM IN EUKARYOTES

Kořený L., Oborník M., Lukeš J.

Original manuscript, will be submitted

The following passage (37 pages) is a manuscript prepared for publication in a scientific journal and it was removed from this version of the thesis that is open to public. It is stored in the archive of the Faculty of Science, University of South Bohemia. The bibliographic information as well as the abstract of this manuscript follows:

Kořený L, Oborník M., Lukeš J. Evolution of heme metabolism in eukaryotes. (unpublished manuscript)
My contribution to this publication is about 90%.

Abstract

This review summarizes current knowledge about the heme metabolism in both heterotrophic and photosynthetic eukaryotes. Particular emphasis is placed on the origin and subcellular localization of enzymes involved in the heme biosynthetic pathway. These two aspects reflect the evolutionary history of eukaryotes as being deeply influenced by endosymbioses and contextual endosymbiotic and non-endosymbiotic horizontal gene transfers. We hypothesize the ultimate causes which could have led to the different spatial organization of heme biosynthesis pathway in organisms with distinct life strategies. Particular attention is paid to eukaryotes, which have lost the ability to synthesize heme.

Study I

TETRAPYRROLE SYNTHESIS OF PHOTOSYNTHETIC CHROMERIDS IS LIKELY HOMOLOGOUS TO THE UNUSUAL PATHWAY OF APICOMPLEXAN PARASITES

Kořený L., Sobotka R., Janouškovec J., Keeling P. J., Oborník M.

Accepted in *Plant Cell*

The following passage (27 pages) is a manuscript recently accepted for publication and it was removed from this version of the thesis that is open to public. The bibliographic information as well as the abstract of this publication follows:

Kořený L, Sobotka R, Janouškovec J, Keeling PJ, Oborník M. 2011. Apicomplexan parasites and photosynthetic chromerids synthesize tetrapyrroles using a homologous non-canonical pathway. *Plant Cell*. (in press).
My contribution to this publication is about 70%.

Abstract

Most photosynthetic eukaryotes synthesize both heme and chlorophyll via a common tetrapyrrole biosynthetic pathway starting from glutamate. This pathway was derived mainly from cyanobacterial predecessor of the plastid and differs from the heme synthesis of the plastid-lacking eukaryotes. Here we show that the coral-associated alveolate *Chromera velia*, the closest known photosynthetic relative to Apicomplexa, possesses a tetrapyrrole pathway that is homologous to the unusual pathway of apicomplexan parasites. We also demonstrate that Chromera is the only known eukaryotic phototroph synthesizing chlorophyll from glycine and succinyl-CoA rather than glutamate. Our data shed light on the evolution of the heme biosynthesis in parasitic Apicomplexa and photosynthesis-related biochemical processes in their ancestors.

Study II

SEQUENCE EVIDENCE FOR THE PRESENCE OF TWO TETRAPYRROLE PATHWAYS IN *EUGLENA GRACILIS*

Kořený L., Oborník M.

Genome Biology and Evolution 3:359–364 (2011)

The following passage (11 pages) is already published in a scientific journal and it was removed from this version of the thesis that is open to public. The bibliographic information as well as the abstract of this publication follows:

Kořený L, Oborník M. 2011. Sequence evidence for the presence of two tetrapyrrole pathways in *Euglena gracilis*. *Genome Biology and Evolution* 3:359–364.
My contribution to this publication is about 90%.

Abstract

Genes encoding enzymes of the tetrapyrrole biosynthetic pathway were searched within *Euglena gracilis* EST databases and 454 genome reads and their 5' end regions were sequenced when not available. Phylogenetic analyses and protein localization predictions support the hypothesis concerning the presence of two separated tetrapyrrole pathways in *E. gracilis*. One of these pathways resembles the heme synthesis in primarily heterotrophic eukaryotes and was presumably present in the host cell prior to secondary endosymbiosis with a green alga. The second pathway is similar to the plastid-localized tetrapyrrole syntheses in plants and photosynthetic algae. It appears to be localized to the secondary plastid, presumably derived from an algal endosymbiont and probably serves only for the production of plastidial heme and chlorophyll. Thus, *E. gracilis* represents an evolutionary intermediate in a metabolic transformation of a primary heterotroph to a photoautotroph through secondary endosymbiosis. We propose here that the tetrapyrrole pathway serves as a highly informative marker for the evolution of plastids and plays a crucial role in the loss of plastids.

Study III

EVOLUTION OF THE HAEM SYNTHETIC PATHWAY IN KINETOPLASTID FLAGELLATES: AN ESSENTIAL PATHWAY THAT IS NOT ESSENTIAL AFTER ALL?

Kořený L., Lukeš J., Oborník M.

International Journal for Parasitology 40: 149–156 (2010)

The following passage (8 pages) is already published in a scientific journal and it was removed from this version of the thesis that is open to public. The bibliographic information as well as the abstract of this publication follows:

Kořený L, Lukeš J., Oborník M. 2010. Evolution of the haem synthetic pathway in kinetoplastid flagellates: An essential pathway that is not essential after all? *International Journal for Parasitology* 40: 149–156.
My contribution to this publication is about 70%.

Abstract

For a vast majority of living organisms, haem is an essential compound that is synthesised through a conserved biosynthetic pathway. However, certain organisms are haem auxotrophs and need to obtain this molecule from exogenous sources. Kinetoplastid flagellates represent an interesting group of species, as some of them lost the complete pathway while others possess only the last three biosynthetic steps. We decided to supplement a current view on the phylogeny of these important pathogens with the expected state of haem synthesis in representative species. We propose a scenario in which the ancestor of all trypanosomatids was completely deficient of the synthesis of haem. In trypanosomatids other than members of the genus *Trypanosoma*, the pathway was partially rescued by genes encoding enzymes for the last three steps, supposedly obtained by horizontal transfer from a c-proteobacterium. This event preceded the diversification of the non-*Trypanosoma* trypanosomatids. Later, some flagellates acquired a b-proteobacterial endosymbiont which supplied them with haem precursors. On the other hand, the medically important trypanosomes have remained fully deficient of haem synthesis and obtain this compound from the host.

Study IV

THE AEROBIC KINETOPLASTID FLAGELLATE *PHYTOMONAS* DOES NOT REQUIRE HEME FOR VIABILITY

Kořený L., Sobotka R., Kovářová J., Gnipová A., Flegontov P., Horváth A., Oborník M., Lukeš J.

Original manuscript, will be submitted

The following passage (21 pages) is a manuscript prepared for publication in a scientific journal and it was removed from this version of the thesis that is open to public. It is stored in the archive of the Faculty of Science, University of South Bohemia. The bibliographic information as well as the abstract of this manuscript follows:

Kořený L, **Kořený L.**, Sobotka R., Kovářová J., Gnipová A., Flegontov P., Horváth A., Oborník M., Lukeš J. The aerobic kinetoplastid flagellate *Phytomonas* does not require heme for viability. (unpublished manuscript)
My contribution to this publication is about 65%.

Abstract

Heme is an iron-coordinated porphyrin that is universally essential as a protein cofactor for fundamental cellular processes. Therefore it is required for viability of virtually all organisms. Here we show that heme is fully dispensable for the kinetoplastid flagellate *Phytomonas serpens*. Analysis of its genome and examination of various cellular processes, for which heme is normally used, revealed that *P. serpens* lacks most of the known hemoproteins and does not require heme for respiration, oxidative stress defence and desaturation of fatty acids. Although heme is still required for the synthesis of ergosterol, its precursor lanosterol is instead incorporated into the membranes of *P. serpens* grown in the absence of heme. Therefore this flagellate has unique metabolic adaptations allowing it to bypass all requirements for heme.

Conclusion

My postgraduate research produced important insights in the evolution of heme biosynthesis in eukaryotes, with profound biological implications. My key findings relate to the elucidation of the evolution of heme metabolism in the major parasitic lineages of eukaryotes. Firstly, I helped to uncover the origin of the unique heme biosynthesis pathway in apicomplexan parasites (e.g. the causing agent of malaria - *Plasmodium*) through a comparative study of their close free-living photosynthetic relatives. Secondly, I described the complex evolution of heme metabolism in kinetoplastid flagellates, a group that includes also important human parasites. One of the most intriguing results was the demonstration that one of these organisms (*Phytomonas*) can survive without heme. My other finding relates to the sequence analyses of tetrapyrrole synthesis genes in *Euglena gracilis*, which represent an intermediate stage in the metabolic transformation of a heterotroph into photoautotroph through a secondary endosymbiosis.