

**EXAMINATION OF THE SUBUNITS OF HYN
HYDROGENASE IN *T. ROSEOPERSICINA* BBS**

Ph.D. Thesis

Lívía Sarolta Palágyi-Mészáros

Supervisors:

Dr. Gábor Rákhely
Prof. Kornél Kovács

Department of Biotechnology, University of Szeged
Institute of Biophysics, Biological Research Centre, Hungarian
Academy of Sciences

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Introduction

Nowadays one of the most important problems of the mankind is the global energy crisis. The fossil energy sources are almost depleted and the demand for energy is growing. The Sun energy is an unexhaustible energy source, which can be utilized by photosynthetic organisms which produce organic carbon molecules from inorganic compounds. However, the collection and storage of Sun-energy are not easy. One of the the obvious solutions is its conversion to enviromental friendly energy carriers, such as biogas, bioethanol or biohydrogen. Taking inot account for the gas house effect of the CO₂ emission, the most promising energy carrier of the future is the hydrogen.

Hyrogenases are the key enzymes in hydrogen metabolism, they catalyses the oxidation of hydrogen and/or reduction of protons. These enzymes represent hopeful tools for economic hydrogen production. However, we must know their proper function and we must modify them for better and more efficient hydrogen production at industrial level.

Hydrogenase enzymes can be categorized into three major groups according to the metal content of the active

centre: [FeFe], [NiFe] and Hmd hydrogenases. Our research group works with [NiFe] hydrogenases.

[NiFe] hydrogenases are heterodimers. The large subunit contains the active site ($\text{NiFe}(\text{CN})_2(\text{CO})$), which is coordinated by conserved cysteines (CxxC motifs). The small subunit harbours [4Fe-4S] and [3Fe-4S] clusters which play role in the electron transport between the large subunit and electron acceptors/donors of the enzyme. The iron in the active center coordinates two CN and one CO residues which function are unknown.

Functional assembly of NiFe hydrogenases is a complex process requiring the concerted action of numerous accessory proteins.

Thiocapsa roseopersicina BBS is a purple sulfur phototrophic bacterium which uses reduced sulfur compounds as electron sources. *T. roseopersicina* cells contain four [NiFe] hydrogenases belonging distinct subclasses. Two of them are membrane-bound (Hup, Hyn) while the other two enzymes are cytoplasmic (Hox1, Hox2).

The Hyn hydrogenase is a bidirectional enzyme which has extraordinary stability under different conditions. The genomic organisation of the *hyn* genes is unusual, since the

genes of the small and the large subunit are separated by two open reading frames, namely *isp1* and *isp2*. According to the *in silico* experiments, *Isp1* is a b-type heme-binding transmembrane protein, while *Isp2* is similar to heterodisulfide- reductases.

The aim of my work was the identification of the redox partners of Hyn hydrogenase, and the investigation of the gene products in the vicinity of the *hyn* genes.

Methods

DNA manipulations and analyses were performed according to the specifications of the manufacturers. Plasmids were transferred into *T. roseopersicina* via conjugation, into *Escherichia coli* by chemical transformation.

The transcriptional organisation of the *hyn* gene cluster was performed with RT-PCR, and I confirmed that *isp1* and *isp2* genes codes for real proteins with transcription analysis. Overproduction of the *Isp2* fused to a His-tag was performed in *E. coli*. Purification was done under denaturing conditions with metal chelate affinity-chromatography.

The function linkage of the Lsp1 and Lsp2 proteins to the Hyn hydrogenase was examined by construction of *in-frame* deletion mutants. The *in vivo* and *in vitro* uptake/evolution activities of the Hyn enzymes of the mutant strains were measured and compared to the wild type and control strains.

The functional role of the conserved amino acids in Lsp2 were examined by site-directed mutagenesis. The conserved amino acids were replaced by another amino acid of distinct character, and the *in vivo* Hyn activities of the Lsp2 point mutant strains were determined and compared.

We used hybrid strategy for sequencing of the *T. roseopersicina* genome. First, I made a shotgun library using the PCR4Blunt kit from Invitrogen (Cat. No.: K7010-01) then a cosmid library with pWEB-TNC kit from Epicentre (Cat. No.: TNC9401) was prepared according to the specifications of the manufacturers. These libraries were sequenced by the automatic Sanger sequencing method. Then, genome sequencing runs were performed by the next generation 454 FLX genome sequencer machine. The sequences obtained from these approaches were combined and assembled.

Results

In my PhD work, the role of the Isp1 and Isp2 proteins in the physiological function of the Hyn hydrogenase was studied in *T. roseopersicina*.

The following results were achieved:

Using RT-PCR analysis, I have demonstrated that the *hynS-isp1-isp2-hynL* genes are located on a single transcript they are transcriptionally coupled.

Applying T7 expression system, I have proved that *isp1* and *isp2* genes code for real proteins.

I have found a gene upstream from the *hynS* gene (*hynH*) which likely codes for a accessory protein involved in the maturation and transport of the Hyn.

I have overproduced the Isp2 protein in heterologous host (*E. coli*). I have purified the protein with affinity-chromatography via a His-Tag fusion partner.

In order to disclose the function of the Isp1 and Isp2, I made several genomic mutants, in which the Isp1 gene (ISP1M) as well as both the Isp1 and Isp2 genes (ISP12M) were deleted. Biochemical characterization of the *in vivo* and *in vitro* Hyn hydrogenase activities of the mutant strains revealed, that the Isp proteins are essential to the proper *in vivo* activity of Hyn. Hereby, I confirmed that the Isp1 and Isp2 proteins are in functional association with Hyn hydrogenase.

The localization of the Hyn hydrogenase in the absence of the Isp proteins has been examined. I have found that deletion of the Isp1 and Isp1,2 had no effect on the localization of the Hyn hydrogenase. According to these results the Isp proteins had no membrane-anchoring role for the Hyn enzyme.

Several conserved amino acids in the Isp2 and its homologous proteins have been found. Applying site directed point mutagenesis, I have replaced these amino acids by residues of completely different property. I have identified few residues which had important or essential role in the *in vivo* Isp2 driven Hyn hydrogenase activity.

I have demonstrated a relationship between the thiosulphate content in the growth media and the hydrogen producing activity of the Hyn. This is an evidence for connection between the Hyn hydrogenase and sulfur metabolism.

I have made genomic shotgun and cosmid libraries from *T. roseopersicina* for determination the genomic sequence of the strain. The next generation 454 method was combined with 30556 sequencing reads obtained from these libraries by Sanger method. This hybrid approach yielded around 98% of the *T. roseopersicina's* total genome.

I have identified numerous genes likely involved in the hydrogen and sulfur metabolism. The gene of the glutathionamide reductase provoked special interest since this enzyme might be a component of metabolic context of the Hyn hydrogenase related to the sulfur metabolism and NAD^+/NADH housekeeping.

Publications

Publication covering the thesis

Palágyi-Mészáros LS, Maróti J, Latinovics D, Balogh T, Klement E, Medzihradszky KF, Rákhely G, Kovács KL. (2009) Electron-transfer subunits of the NiFe hydrogenases in *Thiocapsa roseopersicina* BBS. FEBS J. 276:164-74.

Other publications related to the thesis

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