Concerted regulation of HupSL and Hox1 hydrogenases in *Thiocapsa roseopersicina*

Ph.D. Thesis

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Introduction

Present industrial technologies mostly utilize fossil fuels and the reserves of such energy sources are limited. Furthermore, the combustion of fossil fuels has serious impact on our environment, greenhouse gases get into the atmosphere causing global warming and climate change of the Earth. Therefore, novel, safe and renewable energy sources as well as alternative fuels are needed. The new energy carriers are supposed to be environmentally sound, low-priced, available in large quantities and safely stored.

The most evident energy source is the Sun, we have to utilize it more efficiently in the future. Among the several various possibilities, hydrogen gas (H_2) is one of the most promising candidates, since it is not toxic or carcinogenic, and its combustion results in a sole environment friendly end-product, water. Molecular hydrogen can be produced by chemical processes or by different biological procedures such as biophotolysis of water by photosynthetic algae and cyanobacteria; photofermentation by photosynthetic bacteria or through various two-stage systems combining dark and photofermentation. A wide range of organisms including prokaryotes belonging to the Bacteria and Archaea domains and several eukaryotic algae possess ancient metallo-enzymes able to generate molecular hydrogen. These are the hydrogenase and the nitrogenase enzymes.

The hydrogenase enzymes catalyze the reversible oxidation of the molecular hydrogen. Hydrogenases can produce the clean energy source, or the enzymes can replace the platinum-based electrodes in the fuel cells for hydrogen oxidation. Alternatively, whole-cell systems can be used on the positive pole of the fuel cells to produce electrons through the utilization of biotic substrates or hydrogen as electron source.

The ancient hydrogenases catalyze either proton reduction or hydrogen oxidation *in vivo* in many different metabolic contexts. Various functions are often associated with different cellular localizations of the hydrogenase enzymes. The hydrogenases can be grouped according to their structure, location in the cell and the physiological function as well. The largest and most studied class of hydrogenases is represented by NiFe hydrogenases. H_2 evolution is mostly catalyzed by cytoplasmic enzymes, whereas H_2 uptake is usually done by periplasmic and membrane-associated NiFe hydrogenases. NiFe hydrogenases are classified into four major groups. The H_2 uptake hydrogenases have been shown to play a role in energy conservation through hydrogen recycling. The H_2-evolving hydrogenases produce hydrogen through the reduction of the protons to stabilize the electron homeostasis of the cells by pumping out excessive electrons from the cells. The bidirectional NADP/NAD-reducing, mostly cytoplasmic hydrogenases can either produce or uptake hydrogen depending on the energetic status of the cell. The regulatory hydrogenases control the biosynthesis of Hup-type energy conserving enzymes usually in a hydrogen-dependent manner. Our model organism, the gram-negative photosynthetic purple sulfur proteobacterium, *Thiocapsa roseopersicina*. *T. roseopersicina* was shown to possess four functional NiFe hydrogenases (HynSL, HupSL, Hox1 and Hox2) with differences in their localization, structure and metabolic context. The HynSL and HupSL enzymes are membrane-associated, HynSL was shown to be tightly connected to the sulfur-metabolism of *T. roseopersicina*, while HupSL is considered to play a role in energy conservation under nitrogen-fixing and possibly under thiosulfate-depleted conditions. The Hox1 and Hox2 enzymes are localized in the cytoplasm. The coding sequences of a putative hydrogen-sensing enzyme were identified in *T. roseopersicina*, but the *hupTUV* genes were found to be silent under various tested conditions, therefore it cannot regulate the synthesis of the HupSL

hydrogenase. Expression of the *hupSL* genes was observed to be dependent on the thiosulphate concentration of the growth medium. An increased *in vivo* hydrogen uptake by HupSL was observed under low thiosulfate conditions.

Objectives

The membrane-associated Hup hydrogenases were shown to play important role in energy conservation through hydrogen recycling. The functions of most genes in the *hup* operon are well known. I focused on a previously described but not characterized *orf* residing between the *hupI* and the *hupR* genes. The following specific aims were proposed to investigate the role of the *hupO* gene:

- To perform mutant analysis (in frame *hupO* deletion mutagenesis followed by homologous complementation). To evaluate the *in vivo* and *in vitro* HupSL uptake in the absence or presence of HupO protein.
- *To study* the expression level of *hupSL* structural genes in various *T. roseopersicina* mutant strains.
- To examine the hydrogen dependence of *hupSL* expression.
- To investigate the relationship between the HupSL and further hydrogenases in *T. roseopersicina*, primarily the interaction between the HupSL and Hox1 enzymes.

The Hox1 hydrogenase in *T. roseopersicina* is composed of five functional subunits. The Hox1E subunit completes the Hox1FU diaforase and the Hox1YH hydrogenase subunits. The accurate function of Hox1E is unknown. Similarly, the role of the HoxI in *Ralstonia eutropha* is not characterized. However, it is known that both proteins play a role in the *in vivo* electron transport. It was presumed that these proteins might substitute each other, the presence of the heterologous HoxI subunit might influence the activity of the soluble Hox1 hydrogenase. The specific tasks were as follows:

- To express the heterologous HoxI protein in different *T. roseopersicina* mutant strain.

- Investigation of the effect of HoxI protein on the activity of Hox1 hydrogenase.
- To check the ability of HoxI protein to replace or complement the function of Hox1E.
- To study the global gene expression changes in response to the deletion of *hox1E* and to the addition of heterologous HoxI.

Methods

DNA manipulations were performed according to standard techniques reported in the literature and/or to the protoco**ls** given by the manufacturers. The plasmid constructions were transferred into *E. coli* by transformation, into *T. roseopersicina* via conjugation. In frame deletion mutagenesis was used to investigate the function of the HupO protein. The HoxI protein was expressed from a plasmid in *T. roseopersicina* strains.

The hydrogen evolution and uptake activities of the different strains were measured by gas chromatography *in vivo* and *in vitro* as well.

In order to determine the expression level of specific genes in the selected strains total RNA was purified from the cultures and the expression levels were checked by quantitative reverse transcription coupled Real-Time PCR.

Western blotting technique - with HupL or HoxI primary antibody - was used to compare the protein level in the selected strains under different growth conditions.

Results

A novel *orf* was investigated in the *hupSL* operon in *T. roseopersicina*, now denominated *hupO*. The possible function of HupO was examined.

- Mutant analysis was performed in order to investigate the role of the putative protein product of the *hupO* gene. In frame deletion mutagenesis of *hupO* was applied in different hydrogenase mutant *T. roseopersicina* strains.
- The absence of *hupO* resulted in a significant increase in the *in vivo* and *in vitro* hydrogen recycling activity of HupSL hydrogenase, which was observed exclusively under low-thiosulfate conditions (1 g L^{-1} and 2 g L^{-1}). The introduction of the *hupO* gene in an expression vector fully restored the original low HupSL activity. No significant differences were observed in the very low HupSL hydrogen uptake activity in the presence or absence of *hupO* when high-thiosulfate conditions (4 g L^{-1}) were applied.
- The expression level of the *hupSL* structural genes were strongly upregulated in the Δ*hupO*, Δ*hox1H* mutant strain compared to that measured in Δ*hox1H* strain containing *hupO*. This phenomenon was more evident under low-thiosulfate conditions. A generally high *hupSL* level was observed in all strains containing the Hox1 soluble hydrogenase (irrespective of the presence or absence of *hupO*).
- Based on earlier investigations the expression of the *T. roseopersicina* HupSL hydrogenase was considered independent of the presence or absence of molecular hydrogen. Our experiments corroborated this finding when HupSL synthesis was investigated in presence of *hupO*. However, clear hydrogen dependence of the HupSL synthesis was observed in the Δ*hupO* strain and this phenomenon was the most pronounced when Δ*hox1H* mutant background was applied.

The results suggest a triple control of the HupSL hydrogenase in *T. roseopersicina*. Thiosulfate is the primary regulator: when thiosulfate concentration in the environment is high the HupSL hydrogenase is efficiently repressed in all strains irrespective of the presence or absence of the *hupO* gene and of the presence of further hydrogenases in the cell. Under low thiosulfate conditions, the expression of the HupSL enzyme is elevated in each strain except those lacking the Hox1 hydrogenase. Both the HupSL activity and HupL protein amount are much lower in GB1131 (Δ*hynSL*, Δ*hox1H*) strain compared to strains harboring Hox1 hydrogenase (wild type and Δ*hynSL*), which implies to an as yet uncharacterized connection between Hox1 and HupSL. However, the low Hup activity and expression in GB1131 are significantly increased by the elimination of the *hupO* gene, which supposedly encodes a repressor acting as a second-level regulator. Moreover, hydrogen seems to serve as an additional modulator of Hup functions by influencing the *hup* expression in *hox1* mutant strain when the *hupO* gene coding for a putative repressor is deleted.

The effects of the heterologous HoxI protein on the Hox1 hydrogenase of *T. roseopersicina* was investigated. Expression vector was used to introduce the *hoxI* gene into various *T. roseopersicina* mutant strains. Synthesis of the HoxI protein in *T. roseopersicina* was proven by Western hybridization experiments.

- Results of the comparative hydrogen evolution measurement supported that under high-thiosulfate conditions the strain expressing the *hoxI* gene evolved significantly higher amount of hydrogen *in vivo* than the negative control (4 g L^{-1}). This difference in the hydrogenase activity between was further increased by applying higher thiosulfate concentration (up to 8 g L^{-1}).

- Deletion of *hox1E* gene causes a complete block in the *in vivo* Hox1 hydrogenase activity. This loss-of-function mutation was not complemented by the heterologous *hoxI* gene although these two proteins are considered to play similar role in its host.
- The global gene expression changes were studied in three different strains by whole transcriptome sequencing: the strain containing the complete Hox1 hydrogenase, the *hox1E* mutant strain and the *hox1E* mutant strain harbouring the heterologous *hoxI* gene. The expression of *hox1* and *hox2* structural genes decreased in the Δ*hox1E* strain, while the presence of the *hoxI* gene restored these expressions to the wild-type levels. Similar patterns were observed for the expression levels of the genes of the photosynthetic reaction center and the light harvesting complex. However, two genes showed a unique expression pattern. The NADH dehydrogenase subunit 5 protein and a Hypothetical transmembrane protein coupled to NADH-ubiquinone oxidoreductase chain 5 homolog showed strongly decreased expression both in the *hox1E* mutant strain and in the *hox1E* mutant containing heterologous *hoxI*.

Based on our results we can conclude that the presence of the heterologous HoxI protein increases the hydrogen evolution activity of Hox1 hydrogenase in *T. roseopersicina* under high thiosulfate condition. Interestingly, the heterologous HoxI restores the expression levels of many genes (*hox1* and *hox2* structural genes and the genes of the photosynthetic reaction center and light harvesting complex) showing decreased expression in the *hox1E* deletion strain. However, the *hoxI* cannot restore the *in vivo* Hox1 activity lost in the *hox1E*mutant.

Publication covering the Thesis

Ildikó K. Nagy, Kornél L. Kovács, Gábor Rákhely and Gergely Maróti (2016). HupO is a novel regulator involved in thiosulfate-responsive control of HupSL NiFe-hydrogenase synthesis in *Thiocapsa roseopersicina*. *Applied and Environmental Microbiology* **82:**2039-2049. IF: 3,67

Further publications

Judit Maróti, Attila Farkas, Ildikó K. Nagy, Gergely Maróti, Éva Kondorosi, Gábor Rákhely and Kornél L. Kornél (2010). A Second soluble NiFe enzyme completes the hydrogenase set in *Thiocapsa roseopersicina*. *Applied and Environmental Microbiology* **76:**5113-5123. IF: 3,80

Bernadett Pap, Ádám Györkei, Iulian Z Boboescu, Ildikó K. Nagy, Tibor Bíró, Éva Kondorosi and Gergely Maróti (2014). Temperature-dependent transformation of biogas-producing microbial communities points to the increased importance of hydrogenotrophic methanogenesis under thermophilic operation. *Bioresource Technology* **177:**375-380. IF: 5,09

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