Genes and proteins related to hydrogene metabolism in *Thermococcus litoralis*

Ph.D. thesis

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Introduction

In the course of its development mankind established societies relying on various technical achievements. Operation of these human societies requires energy. The rapid depletion of the fossil fuels draws attention to alternative energy sources and energy carriers. The energy gained from such sources, like the Sun, wind, water or biomass is available in unlimited amounts, although in many cases their use is not economical. To improve the efficienty is the subject of many ongoing research.

The most promising among the alternative energy carriers is hydrogen which is easy to store, transport and the only byproduct of its burning is water. Some microorganisms are able to produce hydrogen with their hydrogenase enzymes. The rapid development of biology and biotechnology in the last decades brought about the possibility to produce hydrogen by using living organism or their isolated components. However, it seems inescapable to influence, modify or reshuffle biological systems in order to serve this goal. This could be achieved by creating engineered photosynthetic organisms that produce large amounts of H_2 by biophotolysis of water. Another alternative is producing H_2 from the harvested biomass of plants or other organic wastes by anaerobic fermentation.

Microorganisms living in extreme environments were discovered few decades ago. They are potential sources of extraordinarily stable proteines that remain active in harsh conditions. Enzymes from hyperthermophilic microbes are already widely used in the biotechnology industry.

T. litoralis is a heterotroph hyperthermophilic archaeon, that was isolated from shallow marine hydrothermal vents. It is a good candidate

for the research of hyperthermophilic microorganisms. It is easy to culture, and contains soluble and membrane-bound heta-stable hydrogenases. It is an interesting microbe from biotechnological view, as during its fermentative growth it utilizes peptides and carbohydrates and produce hydrogene. In our lab a two step procedure was developed in which hydrogene was produced from animal waste. *T. litoralis* was used in the biohydrogen forming step, where it worked much better than the close relative *Pyrococcus furiosus*. Although these two species are very similar to each other there must be some metabolic difference between them. Our experiments suggest that this difference might be in the peptide – hidrogen metabolic pathway. During my work I focused on the components of the hydrogen metabolism.

Our goals were:

- to find accessory genes already known from mesophilic microorganisms, that play an important role in the maturation of hydrogenases in *T. litoralis*.
- to study the isolated accessory genes and their products in heterologous complemenation experiments.
- to find hydrogen evolving membrane-bound hydrogenase in *T. litoralis*.
- to find out, what is the physiological role of the complex coded by the genes located upstream from *hyh-1* operon coding for soluble hydrogenase-1.

Methods

Genes coding for the accessory genes and the hydrogenase structural genes were isolated by screening *T. litoralis* genomic DNA libraries. The isolated genomic regions were subcloned, and the nucleotide sequence was determined. In vitro DNA manipulation and analysis was done according to general practice. The isolated accessory genes were introduced to heterologous hosts and complementation was examined. The transcriptional activity of the *fhl* operon in cells grown on different media was investigated in reverse transcription linked absolute quantification Real-Time PCR experiments. The gene expression was investigated at protein level as well in immunoblotting experiments. Hydrogen evolving and hydrogen uptake measurements were performed, with *T. litoralis* cell fractions. The hydrogenase enzyme was partially purified with Ceramic Hydroxyapatite Chromatography.

Results

1. A ten kilobase and a four kilobase long region of *T. litoralis* genom were isolated and sequenced.

2. Genes coding for HypC and HypD accessory proteins were found in the four kilobase long fragment. After the determination of the transcriptional start site by primer extension a conserved archaeal promoter was identified. The compatibility of the translational signals of *hypCD* genes (ex. ribosomal binding sites) for protein expression in bacterial host was proven.

3. The HypC and HypD proteins were expressed in *E. coli* and *R. eutropha* heterologous hosts in order to perform a functional analyses.

4. The sequence analysis shows that the ten kilobase long region upstream from soluble hydrogenase-1 containes eight genes. Based on *in silico* analysis they code for a complex consisting of a formate-dehydrogenase, and a six-subunit membrane-bound hydrogenase. Every gene is proceeded by tipical ribosomal binding sites and a conserved archaeal promoter can be identified. In RT-PCR experiment we showed that the *fhl* genes form one transcriptional unit.

5. The transcriptional activity of *fhl* operon was investigated in reverse transcription linked Real-Time PCR experiments, using cells grown on different carbon sources and the effect of the presence and absance of sulfur was also checked. Results suggested that the complex coded by the operon was linked to aminoacid metabolism. Its phisiological role is to remove excess reducing equivalents from the cell. In the presence of

sulfur the transcription of the operon was lower than in the absence of S°, in the former case H_2S production acts as an alternative pathway.

6. A protein (FhlB) of the complex was expressed in form of His-tag fusion protein in *E. coli* and purified on nickel chelating column. The purified FhlB was used in immunisation of rabbits, in order to produce polyclonal antibody. The anti-FhlB antibody was purified from rabbit serum and was used in Western hybridization experiments.

7. Western hybridization experiments suggested that the complex was located in the cell membrane, though FhlB and likely the other hydrophilic subunits are weakly associated to the membrane.

8. Hydrogenase activity was detected in the membrane fraction in both uptake and evolution direction. The uptake hydrogenase activity measurement was performed in native polyacrilamide gel as well.

9. FhlB protein was detected in native gel with anti-FhlB in Western hybridization experiments. Its migration position coincided with that of the hydrogenase activity. This and the co-purification of hydrogenase activity and FhlB on CHT chromatography showes that the *fhl* operon encoded hydrogenase and formate-dehydrogenase formes one complex.

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