NAD^+/$NADP^+$ dependent enzymes related to hydrogen and sulfur metabolism in the hyperthermophilic Archaeon, *Thermococcus litoralis*

Ph.D. Thesis

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Introduction

Remarkable part of the permanently increasing energy requirement of the human societies is covered by utilization of fossil fuels which resources are depleting rapidly. In the course of burning of the fossil fuels huge amount of carbon dioxide returns into the atmosphere engendering global climate change through the intensification of the greenhouse effect. Based on these problems one of the most important tasks of mankind is to find alternative, environmental friendly, renewable energy sources and energy carriers.

The most promising candidate among the alternative energy carriers is the molecular hydrogen which is the cleanest fuel because the only product of its burning is water. There are technologies already developed for hydrogen transport, storage and utilization as fuel in vehicles. Hydrogen can be produced by chemical or biological processes. Among the biological strategies an obvious way for the hydrogen production is based on the hydrogenase enzymes. Therefore, all microorganisms which are able to produce hydrogen through their metabolic processes have outstanding biotechnological significance.

Among the hydrogen producing microorganisms the hyperthermophiles are especially important. They are potential sources of extraordinarily stable highly active hydrogenases which could be utilized in both cell free hydrogen producing systems or whole cell applications. Good hydrogen producing hyperthermophilic microbes can be found among the *Thermococcus* and *Pyrococcus* species.

Thermococcus litoralis is an anaerobic heterotroph archaeon which was isolated from shallow marine hydrothermal vents. It is able to utilize both peptides and carbohydrates as sole carbon and energy source. *T. litoralis* produce high amount of hydrogen for elimination of the excess reducing power formed in

the oxidative steps of its fermentative metabolism. In the cells, there are alternative pathways for removing the excess electrons: these include the elemental sulfur reduction which decrease the hydrogen producing capacity of the cells. So far, a soluble hydrogenase (Hyh1) has been characterized in *T. litoralis*, but sulfur reductase is still not described in this archaeon.

Based on its stable enzymes, hydrogen producing capacity, easy and fast cultivation, *T. litoralis* has large biotchnological importance. In our lab, a two-stage system was developed in which biohydrogen was produced from keratin-containing hazardous animal wastes. *T. litoralis* was used in the hydrogen forming step, where it performed much better than the close relative *Pyrococcus furiosus*, which hydrogen metabolism is characterized in detail. On the basis of these results, there might be differences in the hydrogen producing enzymes, the cellular redox balance maintaining metabolic pathways between these archaea. To dislose these differences it was necessary the identification and examination of the still unknown enzymes participating in the hydrogen and sulfur metabolism of *T. litoralis*.

Genetic manipulation methods allowing the efficient examination of the physiological function of proteins are not available for *T. litoralis*. It is difficult to develop genetic systems for hyperthermophilic microorganisms because all processes have to work at the high temperature. Until now, reliable targeted gene disruption system was constructed only for *Thermococcus kodakaraensis* among hyperthermophiles. This system applies a transformant selection method based on the complementation of an uracil auxotroph strain. On the basis of the close relationship between the *Thermococcus* species it was presumed that this genetic method can be applied in *T. litoralis,* as well.

The main aim of my work was to characterize the metabolic processes which form the background of the hydrogen production of *T. litoralis* cells. I planned

- to identify enzymes playing role in the hydrogen and sulfur metabolism of *T. litoralis*,
- to compare these enzymes with those of *P. furiosus* participating in the similar processes, and to determine the differences between the species.
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I also tried to develope a hyperthermophilic genetic system which usable in *T. litoralis*. In this project, my first goal was to isolate uracil auxotroph *T. litoralis* strains as hosts for further transformation experiments.

Methods

Genomic fragments of *T. litoralis* harboring genes of a [NiFe] hydrogenase (*hyh2BDGA*), a sulfur reductase (*nsoABCD*) and pyrimidine biosynthetic enzymes (*pyrE* and *pyrBICD1D2*) were isolated by screening partial genomic DNA libraries and their nucleotide sequences were determined. DNA manipulation and analysis were done according to the standard practice. The transcription start points of the *hyh* and *nso* genes were determined by primer extension experiments. The transcriptional organization of these operons was examined by RT-PCR. The FLAG-tag and Strep-tag II tagged NsoC protein was expressed in *E. coli* and purified by affinity chromatografy according to the manufacturer's instructions. Its native molecular mass was determined by gel filtration chromatography. The UV-visible absorption spectra of the purified protein were recorded. Its cofactor was identified by thin layer chromatography. The role of cysteines of the NsoC enzyme in the catalytic mechanism was investigated by site-directed mutagenesis.

Sulfur reductase, oxygen reductase, hydrogenase and other activity measurements were performed with purified NsoC protein and *T. litoralis* cell fractions. Web-based and local bioinformatic tools were used to analyze sequence data.

Results

My results are summarized in the following points:

1. I isolated and sequenced a 12 kb long genomic region of *T. litoralis* and identified an operon (*hyh2BDGA*) encoding the soluble hydrogenase II enzyme in this archaeon. In the *T. litoralis* DSM5473 strain the enzyme is nonfunctional in consequence of a frameshift mutation in the *hyh2A* gene resulting in a truncated catalytic subunit. I proved that the *hyh2* genes form one transcriptional unit. I determinated the transcriptional start site of the operon and a conserved archaebacterial promoter preceding the genes was identified.

2. I isolated the *hyh2A* gene from *T. litoralis* DSM5474 strain. I showed that the frameshift mutation did not occur in this strain. Hydrogen dependent NAD^+ reduction activity could be measured in the cell extract of the *T. litoralis* DSM5474 strain which confirmed the presence of an active soluble hydrogenase II enzyme in the cells.

3. I identified a gene cluster consisting of four genes (*nsoABCD*) on the 8 kb long chromosomal region upstream from the *hyh2* operon. The *nso* genes are preceded by typical ribosomal binding sites. I determined the transcriptional start site and assigned the promoter elements of the *nso* operon. RT-PCR results proved that all the four genes are located on a single transcript confirming that the gene products likely have linked function. Similar gene cluster could be found only in

Thermococcus kodakaraensis among the Archaea having complete genome sequence. The operon codes for a unusual protein complex, which is most likely of eubacterial origin. Detailed *in silico* analysis of the *nso* gene products suggested the complex to bind nucleotide cofactors and iron-sulfur clusters. I described a domain structure of the NsoC and NsoC homologous enzymes and I proposed a possible mechanism for the evolutionary formation of these proteins.

4. The NsoC subunit fused to tandem FLAG/Strep-tag II was expressed in *E. coli* and purified by affinity chromatography. I demonstrated that the purified NsoC protein contains a non-covalently bound redox active FAD cofactor.

5. I showed that the NsoC protein was a sulfur reductase, which utilized specifically NADPH as electron donor. The enzyme was also able to reduce oxygen, viologen dyes and disulfide bond. I determined the kinetic parameters for the NsoC catalyzed reactions.

6. Systematic site directed mutagenesis study revealed that in NsoC no single cysteine which was essential for catalytic function of the enzyme. This indicated a potentially new catalytic mechanism in this sulfur reductase.

7. I demonstrated that *T. litoralis* cells had NADPH and NADH dependent sulfur reductase actitity, which was located in the cytoplasmic fraction of the cells.

8. I proposed a possible physiological role of the Nso complex: The enzyme might participate in the maintenance of the $NADP^+$/NADPH balance of the cells via sulfur reduction and could be involved in the protection of the cells against oxidative stress.

9. I isolated a 6 kb long chromosomal fragment from *T. litoralis* and identified the gene of the orotate phosphoribosyltransferase (*pyrE*) and an operon (*pyrBICD1D2*) coding for three other enzymes playing role in the *de novo* pyrimidine biosynthesis.

10. I isolated stable uracil-auxotrophic mutant strains of *T. litoralis* using 5-fluoroorotic acid in a positive selection method. I demonstrated that all of these strains possessed the same one basepair deletion in their *pyrE* gene causing the lack of the PyrE enzyme and uracil auxotrophy.

Publications

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