Towards understanding hydrogenase maturation in *Thiocapsa roseopersicina*

Barna D. Fodor

Supervisors:

Prof. Kornél L. Kovács
Dr. Gábor Rákhely

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

Szeged, Hungary

2003

Introduction

Liberation of energy from fossil fuels is accompanied by large-scale pollution, which endangers the environment. Moreover, fossil fuel resources are depleting rapidly. These problems necessitate the search for alternative energy sources and energy carriers. The Sun is a possible source of renewable energy while molecular hydrogen is one of the best candidates for a "clean" energy carrier. The rapid development of biology and biotechnology in the last decades brought about the possibility to produce and utilize hydrogen by using living organisms or their isolated components. However, it seems inescapable to influence, modify or reshuffle biological systems in order to serve this goal. The key components of such systems are hydrogenase enzymes that catalyze the reversible oxidation of molecular H_2. Three major classes are distinguished: metal-free hydrogenases, Fe-only hydrogenases and [NiFe] hydrogenases. The catalytic core of the latter group is typically composed of a heterodimer: an electron transfer small subunit, and a catalytic large subunit. The active site is found in the large subunit and exhibits a unique structure. It is built from a Ni, Fe, CO and two CN^- groups. The diatomic ligands are found on the Fe, and the two metals are coordinated by four conserved cysteins of the large subunit. Not surprisingly the assembly of the active site requires the concerted action of several accessory proteins. The major steps are the biosynthesis of the unprocessed, inactive subunits; the synthesis of the CN^- and CO groups; and insertion of Fe, the diatomic ligands (CN, CO) and Ni into the large subunit (these steps are primarily carried out by the products of the *hyp* genes), and the C-terminal cleavage of the large subunit by a specific protease.

The application of hydrogenases in the sustainable production and utilization of molecular hydrogen is an appealing possibility and stable variants are preferred.

Thiocapsa roseopersicina is a Gram negative, anaerobic photosynthetic, purple sulfur bacterium belonging to the family Chromatiaceae. It harbors at least three [NiFe] hydrogenases (HynSL, HupSL, and HoxYH). One of these (HynSL) has unique properties: it is stable at elevated temperatures (>80°C), resistant to proteases and tolerates O_2. The stability of the *T. roseopersicina* HynSL hydrogenase (coded by the *hynS-isp1-isp2-hynL*

operon) makes it one of the best candidates available for biotechnological applications. Nevertheless, possibility of its utilization is limited by the fact that up to this point an active HynSL enzyme could only be produced in its original host, because of the complicated maturation mechanism, which is a characteristic trait of [NiFe] hydrogenases. Hence, the aim of this study was to set off the identification and characterization of components indispensable in the assembly of an active HynSL hydrogenase (and the other hydrogenases) by different approaches.

Our goals were:

- to create hydrogenase defective mutants and identify genes involved in hydrogenase maturation in *T. roseopersicina*

- to understand how the products of the maturation genes work and function together as a concerted "(protein) assembly machine"

- to develop methods for generating and identifying mutants, and detecting protein-protein interactions

Methods

In vitro DNA manipulation and analysis was done according to the general practice. To introduce recombinant DNA into *T. roseopersicina* a conjugation based gene transfer system was optimized and applied. Site directed and transposon mutagenesis was developed and employed to create mutants. A colony screening method for hydrogenase activity was devised and used to pick transposon generated hydrogenase mutants. Hydrogenase enzyme assay was preformed on various strains to determine enzyme activity. Affinity purification of 6His-, and (FLAG-tag Strep-tag II)- tagged proteins was done according to the manufacturers instructions. Protein analysis was done on native and denaturing polyacrylamide gels. Analysis of proteins co-affinity purifying with tagged proteins was done by MALDI-TOF MS. Web-based and local bioinformatic tools were used to analyze sequence data.

Results

1 I developed a conjugation based gene transfer method for the photosynthetic bacterium *T. roseopersicina*. This is a fundamental tool for the introduction of recombinant DNA into, and genetic manipulation of this organism. Hence, genetic dissection of diverse scientific questions concerning *T. roseopersicina* can be accomplished. (3)

2 I developed a transposon based mutagenesis system for *T. roseopersicina*. This permits the positive selection of mutants in which only a single insertion has occurred. The transposon also tags the mutated genes, thus simplifies the isolation of the corresponding genomic region.

This is the first report on transposon mutagenesis in a purple sulfur photosynthetic bacterium, and should be useful to study various biological processes characteristic of this family of bacteria. (3)

3 I designed and constructed broad-host-range expression vectors with various affinity tags (pMHE* vectors). These facilitate expression of proteins fused to affinity tags, which should simplify detection and purification of these proteins. It was demonstrated that these could be used in various bacteria if a (target) strain specific promoter drove the expression of the protein of interest. These vectors should be useful tools in numerous Gram negative bacteria, when host-specific expression of tagged proteins is necessary. (1)

4 I demonstrated that the pMHE* vectors with strain specific promoters can be used to purify proteins from both the target strain and *Escherichia coli*. Thus, it is needless to construct different expression plasmids when both *E. coli* and another bacterial host are used parallel for protein expression. (1)

5 I proved that tagged target proteins expressed from the pMHE* vectors can be used to affinity purify proteins interacting with the target. This was demonstrated by co-purifying a GroEL-like protein with HupK of *T. roseopersicina* fused with a tandem FLAG-tag Strep-tag II. This approach should be generally useable in various Gram negative bacteria to detect protein-protein interactions. (1)

6 I produced a miniTn5 mutant library of *T. roseopersicina* and developed a screening method to detect hydrogenase mutants. I isolated 6 independent hydrogenase deficient transposon mutants belonging to two mutant classes. The first class of mutations was pleiotropic to all hydrogenases, while members of the second class were HynSL (stable) hydrogenase specific. (2, 3)

7 I identified the *hypF* gene, and proved that its mutation has a pleiotropic effect on all the hydrogenases of *T. roseopersicina*. (3)

8 I demonstrated, by heterologous complementation experiments, that *hypF* genes of different bacteria are interchangeable to a certain extent. These experiments demonstrated that it is

possible to use *hyp* genes of various bacteria while attempting heterologous expression of hydrogenases, but these should be carried out with precaution. (3)

9 I proved that deletion of *isp1* and *isp2* from the *hynS-isp1-isp2-hynL* operon has no effect on the formation of an active HynSL hydrogenase. Hydrogenase activity of membrane fractions was determined from strains harboring plasmids, which carry either the wild type or the *isp1-isp2* deleted version of the operon.

10 I demonstrated directly that the $HypC_2$ and HynL proteins interact during the maturation process leading to an active HynSL hydrogenase. $HypC_2$ fused with a tandem FLAG-tag Strep-tag II was expressed in a $\Delta hypC_2$ *T. roseopersicina* strain. The tagged protein complemented the mutation. HynL co-purified with the tagged $HypC_2$. Hence, $HypC_2$ is proposed to be a HynL specific chaperone. (1)

11 I showed that the hydrogenase accessory protein, HupK, interacts with a GroEL like protein. The potential role of the GroEL like protein in hydrogenase maturation is discussed. (1)

Publications

Papers related to the thesis

1. Fodor, B. D., Á. T. Kovács, R. Csáki, É. Hunyadi-Gulyás, É. Klement, G. Maróti, L. S. Mészáros, K. F. Medzihradszky, G. Rákhely, and K. L. Kovács. 2003. Modular broad-host-range expression vectors for single protein and protein complex purification. Submitted to Appl. Environ. Microbiol.

2. Maróti, G., B. D. Fodor, G. Rákhely, Á. T. Kovács, S. Arvani, and K. L. Kovács. 2003. Accessory proteins functioning selectively and pleiotropically in the biosynthesis of [NiFe] hydrogenases in *Thiocapsa roseopersicina*. **Eur. J. Biochem.** 270:2218-2227.

3. Fodor, B., G. Rákhely, Á. T. Kovács, and K. L. Kovács. 2001. Transposon mutagenesis in purple sulfur photosynthetic bacteria: identification of *hypF*, encoding a protein capable of processing [NiFe] hydrogenases in alpha, beta, and gamma subdivisions of the proteobacteria. **Appl. Environ. Microbiol.** 67:2476-2483.

Other papers

4. Rákhely, G., A. T. Kovács, G. Maróti, B. D. Fodor, Gy. Csanádi, D. Latinovics, and K. L. Kovács. 2003. A cyanobacterial type, heteropentameric NAD^+ reducing [NiFe] hydrogenase in the purple sulfur photosynthetic bacterium, *Thiocapsa roseopersicina*. Submitted to Appl. Environ. Microbiol.

5. Kovács K.L, B. D. Fodor, Á. T. Kovács, Gy. Csanádi, G. Maróti, J. Balogh, S. Arvani, and G. Rákhely. 2002. Hydrogenases, accessory genes and the regulation of [NiFe] hydrogenase biosynthesis in *Thiocapsa roseopersicina*. **Int. J. Hydrogen Energy** 27:1463-1469.

6. Kovács, K. L., C. Bagyinka, L. Bodrossy, R. Csáki, B. Fodor, K. Győrfi, T. Hanczár, M. Kálmán, J. Ősz, K. Perei, B. Polyák, G. Rákhely, M. Takács, A. Tóth, and J. Tusz. 2000. Recent advances in biohydrogen research. **Pflugers Arch.** 439:R81-R83.

7. Dahl, C., G. Rákhely, A. S. Pott-Sperling, B. Fodor, M. Takács, A. Tóth, M. Kraeling, K. Győrfi, Á. Kovács, J. Tusz, and K. L. Kovács. 1999. Genes involved in hydrogen and sulfur metabolism in phototrophic sulfur bacteria. **FEMS Microbiol. Lett.** 180:317-324.