# Insights into the assembly of the NiFe hydrogenase enzymes

Ph.D. Thesis

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# Introduction

Some photosynthetic organisms have the capability to produce molecular hydrogen by converting solar energy into this clean energy source. Hydrogen can be burnt, high amount of clean energy can be generated, and the only byproduct is water. Certain organisms contain ancient enzymes, which can evolve hydrogen from protons and electrons. These enzymes are the hydrogenases, which catalyze the oxidation of molecular hydrogen into protons and electrons, and the reverse reaction, the reduction of protons to yield molecular $H_2$.

Hydrogenase enzymes can be classified by several properties. They differ in their physiological role and localization, but the basic principle in the classification is the structure, the metal content of the active centre of the enzymes. Three main classes can be distinguished: hydrogenases free of redox-active metals, [FeFe] hydrogenases and [NiFe] hydrogenases. The [NiFe] hydrogenases compose the largest group. The catalytic core protein is heterodimeric. This minimum protein structure of the [NiFe] hydrogenases is necessary for the hydrogen evolution/oxidation. The large subunit is about 64 kDa, it contains the binuclear metallocenter, which is the active site of the enzyme. Four cysteines anchor the active center, they are arranged in conserved motifs, two of them at the N-terminal region and two cysteines at the C-terminus. Although the [NiFe] and the [FeFe] hydrogenases have completely different structures and are evolutionary unrelated, they share a common

feature, namely the presence of endogenous CO and CN- ligands bound to the Fe atom in the active site. The small subunit of 30 kDa binds a conserved [4Fe-4S] cluster located in the proximity of the active site. Two additional [Fe-S] clusters are usually also located in the small subunit. These clusters are responsible for transferring electrons to the surface of the enzyme, and are thought to interact with redox partners.

Considering the complexity in function and localization, in addition to the structural sophistication of [NiFe] hydrogenases and their active sites, it is not surprising that many genes are required for the biosynthesis of these enzymes. The subunits coded by the structural genes have to undergo modification processes mediated by the so-called accessory proteins. This molecular assembly requires at least 10 auxiliary proteins. Some of them are needed for the maturation of all hydrogenase enzymes in the cell, these are the **hy**drogenase **p**leiotropic (Hyp) accessory proteins, while others are specific for only one hydrogenase in the host organism.

*Thiocapsa roseopersicina* BBS harbors at least 3 functioning hydrogenases. All three enzymes show the characteristics of the [NiFe] hydrogenases, two of them are attached to the periplasmic membrane (HynSL, HupSL), while the third one is localized in the cytoplasm (HoxYH). The genes showing similarity to sequences coding for the regulatory hydrogenases (HupUV/ HoxBC) of other bacteria were identified in *T. roseopersicina* (*hupUV*). No hydrogen-dependent regulation was observed in this strain. The lack of

expression of the *hupUV* genes explains the hydrogen independent phenotype.

The basic principles of the [NiFe] hydrogenase maturation should be valid in the case of the hydrogenases of *T. roseopersicina*, as the primary structures of the enzymes are well conserved.

My main aim was to examine these biosynthetic processes, to identify the protein partners, and to establish the roles of these accessory proteins during the hydrogenase biosynthesis. The individual steps of this process probably have an accurate order in time as well, but this is not known in detail to date in any microorganisms.

# Methods

DNA manipulations and analyses were performed according to standard techniques and the specifications of the manufacturers. Plasmids were transferred into *T. roseopersicina* via conjugation. My main tools were the different types of mutation analyses, which give the opportunity for specific examinations of the different hydrogenases, and can block the maturation process in various stages. Transposon mutagenesis was applied to search for the auxiliary genes. The organization of the accessory genes was examined by RT-PCR and β-galactosidase reporter gene fusion activity measurements. Site directed mutagenesis (in most cases in frame) was used to study the specificity of the accessory proteins. As a first approach hydrogenase activity measurements (both in uptake

and evolution direction) were used to characterize the various mutant phenotypes. Affinity purification of FLAG-tag and Strep-tag II-tagged accessory proteins was done according to the manufacturers instructions. Protein analysis was done on native and denaturing polyacrylamide gels. Proteins co-purified with tagged proteins were determined by MALDI-TOF MS.

# Results

In brief, the behaviour of several accessory proteins was understood in my work, these proteins are selectively or pleiotropically involved in the biosynthesis of the various [NiFe] hydrogenases in *T. roseopersicina*. The following statements have been established on the basis of my results:

I identified several accessory proteins participating in the biosynthesis of the [NiFe] hydrogenases by using transposon mutagenesis. The analysis of the M442 mutant strain revealed the *hupK*- $hypC_1$-*hypD*-*hypE* genes. The transposon inserted into the *hynD* gene in the M1250 mutant strain, the $hypC_2$ gene was near the *hynD* in opposite orientation.

I have found the missing *hypA* and *hypB* basic accessory genes by searching in the partial genome sequence of *T. roseopersicina*.

I examined the regulation of the accessory genes, the *hypC*$_1$-*hypD* genes transcribed together with the *hupK* gene according to the RT-PCR analysis, but independent transcription of *hypC*$_1$-*hypD* also exists, which was proven by homologous complementation experiments.

The co-transcription of the *hoxW* with the *hoxEFUYH* and *hupD* with *hupSLC* genes, respectively, was determined using RT-PCR. HoxW and HupD are specific endopeptidases participating in the last step of hydrogenase maturation. The FNR system regulates the expression of the *hynSL* structural genes but the *hynD* gene, coding for the endopeptidase of HynSL, is not regulated by this global anaerobic regulator.

I performed the mutation analyses for the endopeptidase coding genes. In frame mutants were created in the cases of *hynD* and *hupD* genes, while an antibiotic resistance cassette was inserted into the *hoxW* gene. As one would expect on the basis of the few analogous cases in other bacteria, the endopeptidases are completely specific, the HynD cuts the C-terminal end of the HynL subunit, the HupL plays role only in the maturation of the HupL subunit, and the HoxW is neccessary only for the assembly of the Hox hydrogenase.

I created *ΔhupK* in frame deletion mutant strains in *T. roseopersicina* in order to examine the specificity and possible role of this protein. The mutation affected seriously the membrane associated [NiFe] hydrogenases, while the soluble hydrogenase retained almost all of its activity. Homologous complementation with the *hupK* gene restored all hydrogenase activities.

I mutated the $hypC_1$ (in frame deletion) and $hypC_2$ genes in several hydrogenase backgrounds. This made possible the investigation of the specificities of these chaperon-like maturation peptides. Unlike the findings in the *E. coli* model, both HypC-s have integral role during the maturation of all three [NiFe] hydrogenases in *T. roseopersicina*. Complementation experiments proved the role of the HypC-s in the observed mutational effects.

I used the pMHE broad host range expression vector system developed in our lab for purifying the accessory proteins from *T. roseopersicina*. I purified the tagged HupK and HypC proteins in active form, and I have identified some important maturation complexes. The $HypC_2$ interacts at least with the HynL, and direct contact was detected between $HypC_1$ and the large subunit of the soluble hydrogenase (HoxH).

I set up a model for the maturation of [NiFe] hydrogenases in phototrophic sulfur bacteria especially in *T. roseopersicina,* based on literature and my experimental data. The main discrepancy from the *E. coli* model was observed in the role of HypC-s. These chaperon-like proteins cannot substitute each other during the maturation process, I propose distinct roles for $HypC_1$ and $HypC_2$.

# Publications

## Publications covering the thesis


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

**Fodor, B. D., Kovács, Á. T., Csáki, R., Hunyadi-Gulyás, É., Klement, É., Maróti, G., Mészáros, L. S., Medzihradszky, K. F., Rákhely, G. and. Kovács, K. L. (2004)** Modular broad-host-range expression vectors for single protein and protein complex purification. *Appl. Environ. Microbiol.* **70**:712-721

**Kovács, K. L., Kovács, Á. T., Maróti, G., Mészáros, L. S., Balogh, J., Latinovics D., Fülöp, A., Dávid, R., Dorogházi E. and Rákhely, G. (2005)** The hydrogenases of *Thiocapsa roseopersicina*. *Biochemical Society Transactions* **33**: 61-63.

**Rákhely, G., Kovács, Á. T., Maróti, G., Fodor, B. D., Csanádi, G., Latinovics, D. and Kovács, K. L. (2004)** Cyanobacterial type, heteropentameric, $NAD^+$ reducing [NiFe] hydrogenase in the purple sulfur photosynthetic bacterium, *Thiocapsa roseopersicina. Appl. Environ. Microbiol.* **70**:722-728.

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

## Other publications

**Kovács, Á. T., Rákhely, G., Browning, D. F., Fülöp, A., Maróti, G., Busby, S. J. W. and Kovács, K. L. (2005)** An FNR-type regulator controls the anaerobic expression of Hyn hydrogenase in *Thiocapsa roseopersicina. J Bacteriol*. (in press)

**Kovács, Á. T., Rákhely, G., Balogh, J., Maróti, G., Fülöp, A. and Kovács, K. L. (2005)** Anaerobic regulation of hydrogenase transcription in different bacteria. *Biochemical Society Transactions* **33**: 36-38.

**Kovács, K. L., Bagi, Z., Bálint, B., Balogh, J., Dávid, R., Fodor, B. D., Csanádi, G., Hanczár, T., Kovács, Á. T., Latinovics, D., Maróti, G., Mészáros, L., Perei, K., Tóth, A. and Rákhely, G. (2004)** Microbial hydrogen metabolism and environmental biotechnology. In: Environmental Biotechnology (Ed. W. Verstraete) Taylor and Francis, London. pp. 155-158.

**Maróti, G. (2004)** Maturation of hydrogenase enzymes in *Thiocapsa roseopersicina*. *Acta Biol. Szegediensis* **48**: 67

**Kovács, K.L., Bagi, Z., Bagyinka, Cs., Bodrossy, L., Csáki, R., Fodor, B., Hanczár, T., Tusz, J., Kálmán, M., Klem, J., Kovács, Á.T., Jian, L., Magony, M., Maróti, G., Perei, K., Polyák, B., Arvani, S., Takács, M., Tóth, A. and Rákhely, G. (2000)** Biohydrogen, biogas, bioremedition. *Acta Biol. Debrecina* **22**:47-54.

**Maróti, G., Rákhely, G. and Kovács, K. L. (2002)**
Hidrogéntermelő biogyárak. (*Hungarian*) Élet es Tudomány OTKA cikkpályázat I. hely.

## Co-author’s disclaimer declaration

I (undersigned) declare, that I am familiar with the candidate’s thesis points, I have not used the scientific achievements declared in his thesis points to acquire any scientific degree, and I take cognizance, that I will not use those for such reason in the future.

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