Structure-function analysis of membrane-associated Hyn [NiFe] hydrogenase in *Thiocapsa roseopersicina* BBS

Ph.D. Thesis

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Introduction

Present industrial technologies mostly utilize fossil fuels, thus the reserves of such energy sources are getting more limited as it is also indicated by their growing prices. Furthermore, their combustion has serious impact on our environment via greenhouse effect, air pollution and water contamination. Therefore novel, safe and renewable energy sources as well as altenative fuels are needed. Among the several various possibilities, hydrogen gas (H_2) is one of the most promising candidates, since its combustion results in only environment friendly species, namely water. Molecular hydrogen can be produced biologically by hydrogenase or nitrogenase enzymes harboured by several microorganisms.

Hydrogenases catalysing the reversible heterolytic cleavage of molecular hydrogen are the key enzymes of the hydrogen metabolism. The majority of the known hydrogenases belongs to either [NiFe] or [FeFe] hydrogenases containing FeS-clusters, but there are also FeS-cluster-free, so-called [Fe] hydrogenases in some methanogen bacteria. The core of the [NiFe] hydrogenase consists of a small and a large subunit. The former is responsible for the electron transfer between the active centre and the *in vivo* electron donor/acceptor of the enzyme via FeS-clusters, while the large subunit harbours the binuclear, NiFe active site where the heterolytic cleavage of the molecular H_2 takes place. The active site contains 2 CN^- and 1 CO ligand bound to the Fe ion.

Our model organism, *Thiocapsa roseopersicina* BBS is a Gram-negative, purple sulphur photosynthetic bacterium belonging to

the *Chromatiaceae* family. It contains four functional [NiFe] hydrogenases (Hyn, Hup, Hox1, Hox2) differing in their *in vivo* function, localization and composition.

The Hyn hydrogenase is a bidirectional enzyme with remarkable stability; therefore it can be a good candidate for future biotechnology applications. In order to force either the microbes or their isolated enzymes to produce large amount of hydrogen continuously, we have to understand the function of those structural motifs which are generally found in [NiFe] hydrogenases and which might be responsible for the adequate operation of the enzyme during the catalytic cycle.

Site-directed mutagenesis is one of the possible methods for studying the structure-function relationships in the enzyme. Applying this method, we are able to examine the function of one conserved amino acid or an entire motif in the enzyme.

One of my aims was to elucidate the function of a His-rich region (HxHxxHxxHxH) occurring in the large subunit of almost all membrane associated [NiFe] hydrogenases. The second histidine of this motif has been already reported in the literature as a member of a proton transfer pathway. Accordingly, I explored the relationship between this conserved region and an alternative proton transfer pathway.

Methods

DNA manipulations and analyses were performed according to standard techniques reported in the literature and/or to the protocols given by the manufacturers. Plasmids were transferred into *E. coli* by transformation, into *T. roseopersicina* via conjugation.

Function of the histidine-rich motif and the proposed proton transfer pathway was studied by site-directed mutagenesis. In all of these experiments, the HynSL hydrogenase was the model enzyme. The mutant constructions were conjugated into a triple mutant *T. roseopersicina* strain, which is unable to express the Hyn, Hup and Hox1 from the genome, therefore it was possible to study the specific effects of the mutations. Hydrogen uptake and evolution activities of metabolically active cultures and disrupted cells were measured and used to characterize the phenotypes of various mutants. The proteolytic stability of the Hyn mutants was checked by Western hybridization. The relationship between the submolecular pathways, essential for the catalytic mechanism of hydrogenases, and the histidine-rich region, typical for the membrane-bound enzymes were studied by bioinformatics. This method was used to identify and characterize the hydrogen bonding partners of the most relevant amino acid (His104) of the histidine-rich motif as well.

Results

Crystal structures of several periplasmic membrane-associated [NiFe] hydrogenases mostly belonging to the *Desulfovibrio* genus were used to study highly conserved regions and conserved amino acids typical for [NiFe] hydrogenases, which might have important roles during catalytic cycle of the enzyme. One of the aims of this work was to map other conserved sequences typical for the [NiFe] hydrogenases using *T. roseopersicina* HynSL enzyme as a model. A highly conserved His-rich region (HxHxxHxxHxH) was found and the examination of this motif was performed by site-directed mutagenesis. The following statements could be established based on my results:

HxHxxHxxHxH motif occurs in the large subunit of almost all known membrane-bound [NiFe] hydrogenases however, only the second and the fourth histidines of this motif are conserved in the cytoplasmic [NiFe] hydrogenases.

Site-directed mutagenesis of the His-rich region of the *T. roseopersicina* membrane-attached HynSL hydrogenase disclosed that the enzyme activity was significantly affected only by the replacement of the His104 residue.

The substitution of the conserved amino acids of the His-rich motif by alanine did not change the localization of the enzyme.

Computational analysis of the available crystal structures of membrane-associated [NiFe] hydrogenases revealed a network of conserved amino acids - with the participation of His104 - connected by strong hydrogen bonds. The properties of this molecular chain suggested that it may be a possible candidate for a proton transfer pathway during the enzyme catalysis.

The mutation of the conserved amino acids of the proposed proton transfer pathway had influence on the activity of the Hyn hydrogenase.

Based on the results of the Western hybridization experiments, it has been confirmed that the substitution of either amino acids of the proposed proton channel (Asp103, His104) does not change the amount of the mutant HynL protein compared to that of the wild type. These results clearly demonstrated that the change of the appropriate amino acids had no effect on the proper biosynthesis of the active enzyme.

It has been pointed out that the first amino acid of the proposed proton transfer pathway (Arg487) has dual function in [NiFe] hydrogenases. The replacement of this amino acid has influence not only on the catalytic activity, but also on the biosynthetic processes of the enzyme. Arg487 probably might perturb the coordination sphere of the Fe ion in the metal center, since it is capable to form two hydrogen bonds with CN^- ligands of the active site Fe. Thus, it can affect the biosynthesis and thereby the enzyme activity of [NiFe] hydrogenases.

In this work I have proposed a novel proton transfer pathway partly overlapping with a previously suggested proton transfer route. I provided an alternative way how protons can be transferred between the active site and the surface of the enzyme.

Our model compared to the previous suggestions mostly based on theoretical calculations, indicates that this possible proton transfer pathway is located on the opposite side of the large subunit relative to the position of the small subunit (where the electron transfer takes place).

It is the first time that the conclusions of a molecular modelling study have been successfully confirmed by experimental results using model organism distinct from the well-known *Desulfovibrio*-type ones. The general features of the proposed novel proton channel could be detected in the *Allochromatium vinosum* structure as well, the close relative organism of the *T. roseopersicina*.

Publications

Publications covering the thesis

Emma Szőri-Dorogházi, Gergely Maróti, Milán Szőri, Andrea Nyilasi, Gábor Rákhely, Kornél L. Kovács (2012) Analyses of the large subunit histidine-rich motif expose an alternative proton transfer pathway in [NiFe] hydrogenases, *Plos One* (**in press**); IF(2010): 4,411

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Further publications

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