A hydrogen-sensing multiprotein complex controls aerobic hydrogen metabolism in Ralstonia eutropha

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Abstract

H₂ is an attractive energy source for many microorganisms and is mostly consumed before it enters oxic habitats. Thus aerobic H₂-oxidizing organisms receive H₂ only occasionally and in limited amounts. Metabolic adaptation requires a robust oxygen-tolerant hydrogenase enzyme system and special regulatory devices that enable the organism to respond rapidly to a changing supply of H₂. The proteobacterium Ralstonia eutropha strain H16 that harbours three [NiFe] hydrogenases perfectly meets these demands. The unusual biochemical and structural properties of the hydrogenases are described, including the strategies that confer O₂ tolerance to the NAD-reducing soluble hydrogenase and the H₂-sensing regulatory hydrogenase. The regulatory hydrogenase that forms a complex with a histidine protein kinase recognizes H₂ in the environment and transmits the signal to a response regulator, which in turn controls transcription of the hydrogenase genes.

Introduction

H₂ is an attractive energy source for many microorganisms including bacteria, archaea and lower eukaryotes. Most of the H₂ that is produced during fermentation is consumed by anaerobic microbes. Organisms that thrive in oxic environments probably receive H₂ only occasionally and it is not at all plentiful. For these mostly facultative chemolithoautotrophs, it is beneficial to use alternative organic substrates. To survive and compete successfully in oxic habitats, facultative chemolithoautotrophs have to meet two basal requirements: (i) their H₂-oxidizing enzyme system has to tolerate O₂ even during catalysis, and (ii) a regulatory device is necessary to guarantee sensitive detection of H₂ and rapid adaptation to changing supply of organic material.

The β-proteobacterium Ralstonia eutropha strain H16 (isolated as Hydrogenomonas eutropha, renamed as Alcaligines eutrophus and recently reassigned as Wautersia eutropha [1]) represents an excellent model to study aerobic adaptation of H₂ metabolism on both the enzymatic and regulatory levels. The high metabolic flexibility of R. eutropha is reflected by a complex genome encompassing 7.40 Mbp of DNA distributed on three independent replicons: Chromosome 1 (4.05 Mbp), chromosome 2 (2.90 Mbp) and megaplasmid pHG1 (0.45 Mbp; [2]). H₂ metabolism is a plasmid-borne character in R. eutropha H16. pHG1 harbours two well-defined hydrogenase operons. (i) The MBH (membrane-bound hydrogenase) operon encodes structural and accessory functions for a membrane-bound hydrogenase, for metallocentre assembly and regulation [3,4]. (ii) The SH (soluble hydrogenase) operon codes for a soluble hydrogenase, accessory functions and an incomplete set of metallocentre assembly proteins [5–7]. Surprisingly, sequence analysis uncovered an additional hydrogenase gene cluster spaced between the MBH and SH operons [2]. There is no evidence that these genes are actively expressed. Although the role of the additional hydrogenase remains obscure, it is interesting to note that the putative additional hydrogenase of R. eutropha shows highest sequence similarity with the putative [NiFe] hydrogenase in the Gram-positve Streptomyces avermitilis. The accessory proteins resemble cyanobacterial counterparts. Thus it seems as if the additional hydrogenase gene cluster is of alien origin.

The SH deviates in many features from standard [NiFe] hydrogenases

R. eutropha has two [NiFe] hydrogenases that are involved in energy conservation from H₂ (Figure 1). The heterodimeric periplasmically orientated MBH is anchored to the membrane by a b-type cytochrome and primarily involved in the generation of a proton gradient. The SH belongs to a subfamily of multimeric [NiFe] hydrogenases [8] and resides in the cytoplasm. The enzyme is composed of the dimeric NiFe-centre-containing hydrogenase module (HoxHY) and the FMN-accommodating NADH dehydrogenase moiety (HoxFU), which confers the ability to reduce directly NAD⁺ at the expense of H₂, thus delivering reducing equivalents to the cell.

It has been known for decades that the SH not only differs in its oligomeric state but also in several biochemical features from standard [NiFe] hydrogenases, e.g. the enzymes...
from Desulfovibrio gigas or Allochromatium vinosum. (i) The SH is catalytically active in the presence of O₂ and CO, (ii) harbours an NiFe site that shows no redox changes and (iii) is rapidly activated by NADH and scarcely activated by H₂ alone [9,10].

The list of uncommon features has been extended more recently. FTIR (Fourier-transform infrared) spectroscopy indicated a modified NiFe site [(RS)₂(CN)Ni(μ-RS)-Fe(CN)₃(CO), R = Cys] in the SH with two extra cyanide (CN⁻) groups, one assigned to the Fe and one assigned to the Ni ion (Figure 1; [11]). Chemical analysis and FTIR spectra of ¹⁵N-enriched SH preparations corroborated a modification of the SH NiFe active site. Reductive treatment of the SH led to selective removal of the Ni-bound CN⁻ ligand. This process was accompanied by the induction of O₂ sensitivity pointing to a protective role of one of the CN⁻ groups [12].

Genetic data support the existence of a modified NiFe site in the SH. As elegantly shown for Escherichia coli, metallocentre assembly into hydrogenase is a protein-assisted process requiring at least six Hyp proteins (HypA, HypB, HypC, HypD, HypE and HypF, [13]). A few bacteria, interestingly those that metabolize H₂ under aerobic conditions, harbour an additional hyp gene, designated as hypX [14,15]. Due to an N-terminal N₁₀ formyltetrahydrofolate-binding motif, a carbonyl-group donating function was discussed for HypX [14]. We have investigated the phenotype of a hypX mutation in R. eutropha H16 in more detail. Lithoautotrophic growth of the hypX mutant was severely inhibited by O₂. Even at low concentrations of 5% O₂, the doubling time was significantly increased. Purified SH from HypX⁻ cells was active under anaerobic assay conditions but irreversibly destroyed in the presence of O₂. FTIR spectra of the mutant protein identified the loss of one CN⁻, specifically that was assigned to Ni [16]. These observations are in accordance with the assumption that the Ni-bound CN⁻ plays a crucial role in the O₂ tolerance of the SH.

Two other discoveries, inserted in the renewed model of the SH (Figure 1), may shed more light on the reaction mechanism. (i) Evidence was presented recently for a second FMN cofactor (FMN-a) located in the hydrogenase module HoxHY. FMN-a appears to communicate with the NiFe active site and is discussed as a hydride acceptor [17]. (ii) Not only a cofactor but also an additional subunit was found in a hexameric preparation of the SH (HoxHYFUI2) isolated under conditions that deviate from the method that had been formerly applied [18]. The subunits were identified as the dimeric product of the SH accessory gene hoxI, which turned out to be identical with the so-called B-protein that was previously demonstrated to be co-expressed with the SH at high level [19]. In contrast with the tetramer, the hexameric form of the SH can not only be activated by NADH but also with NADPH, indicating that the HoxI dimer provides
a binding site for an alternative nucleotide whose interaction with the SH may be of benefit for catalysis (T. Burgdorf, E. van der Linden, M. Bernhard, Q.Y. Yin, J.W. Back, A.O. Muijsers, C.G. de Koster, S.P.J. Albracht and B. Friedrich, unpublished work).

**Regulation of hydrogenase gene transcription by H\(_2\) sensing**

The two hydrogenase operons of *R. eutropha* H16 are coordinately regulated and transcribed from \(\sigma^{54}\)-dependent promoters in response to \(\text{H}_2\) and the quality of the carbon source (Figure 1; [20]). On fast growth-supporting substrates like succinate, box gene transcription is repressed even in the presence of \(\text{H}_2\), whereas on poor substrates like glycerol induction occurs [20]. We know little about the molecular mechanism of the global carbon control, which is superimposed on \(\text{H}_2\) induction. Therefore this survey will focus on the \(\text{H}_2\)-triggered transcriptional control. A similar regulatory system has been described in the \(\text{H}_2\)-oxidizing phototroph *Rhodobacter capsulatus* [21] and the nitrogen fixer *Bradyrhizobium japonicum* [22].

The principal player in the regulatory cascade of *R. eutropha* H16 is HoxA, a response regulator of the NtrC family that carries a central ATPase domain necessary for the activation of \(\sigma^{54}\) RNA polymerase. A conserved aspartate residue in the receiver module of HoxA indicates modification through phosphorylation. The cognate histidine protein kinase is encoded by boxJ. A PAS signature in the input domain of HoxJ points to the processing of a redox signal [23]. A phosphoryl group transfer between HoxJ and HoxA could be demonstrated with purified components using \(\gamma\)-\[^{32}\text{P}\]ATP as phosphoryl donor (M. Forgber, O. Lenz and B. Friedrich, unpublished work).

The core of the \(\text{H}_2\) sensing system consists of a \([\text{NiFe}]\) hydrogenase encoded by boxB and boxC, designated as RH (regulatory hydrogenase) [23].

**Characteristics of the sensor hydrogenase**

With the aid of a homologous overexpression system, the RH was purified from the cytoplasm of *R. eutropha* H16 [23] and its major biochemical characteristics are as follows: the RH differs from standard \([\text{NiFe}]\) hydrogenases in several properties. It forms a tetramer consisting of two heterodimeric units. Although the RH is able to oxidize \(\text{H}_2\) with dyes as electron acceptor, the turnover rate is very low. The interacting partner of the RH is the protein kinase HoxJ [24].

Synthesis of the \([\text{NiFe}]\) site relies on the action of the Hyp proteins HypA, HypB, HypC, HypD, HypE and HypF but not on HypX [25]. Unlike in most cases proteolytic processing of the large subunit does not occur. The RH was identified as an Ni-containing hydrogenase, which according to FTIR studies accommodates a standard NiFe centre \([\text{(RS)}_2\text{Ni}(\mu-\text{RS})_2\text{Fe(CN)}_2(\text{CO})]; \text{R} = \text{Cys}]\) [26]. On the basis of X-ray absorption spectroscopy, the RH undergoes \(\text{H}_2\)-induced structural changes at the Ni site [27]. The RH shows an EPR-detectable Ni-C state after reduction with \(\text{H}_2\) that is similar to the Ni-C state of standard \([\text{NiFe}]\) hydrogenases. In contrast with the latter group of enzymes, Ni-A or Ni-B signals are missing in the RH. The protein seems to be frozen in the active state and does not require reductive activation to get ready for \(\text{H}_2\) binding [26]. This property is compatible with the function of a sensor that has to be on constant alarm to detect \(\text{H}_2\) immediately in the environment. Advanced spectroscopy (ENDOR and HYSCORE) was applied to obtain further structural information of the Ni-C state. For the first time evidence was presented that an exchangeable proton resides in a bridging position between the Ni and Fe, assigned to a formal hydride ion. The Ni-L state was formed on illumination and the strong hyperfine coupling was lost, indicating a cleavage of the hydride–metal bond [28].

The RH functions in the presence of \(\text{O}_2\) and \(\text{CO}\), but obviously uses a different strategy than the SH to acquire these abilities. Crystallographic studies of standard hydrogenases have shown that the Ni-Fe centre is deeply embedded inside the protein. Xenon-labelling experiments probed channels connecting the catalytic site with an external medium [29]. The structure of these gas channels appears to be highly conserved.

A possible route for molecular \(\text{H}_2\) ends near a vacant Ni-co-ordination site. The flanking positions are occupied by Val-67 and Leu-115 (co-ordinates of the hydrogenase large subunit HydB from *D. gigas*). Alignment and modelling of amino acid sequences of \(\text{H}_2\)-sensing proteins identified more bulky residues (Ile-62 and Phe-110 of the RH large subunit HoxC) at these positions, possibly affecting the access of larger gas molecules to the Ni-Fe site [30]. The hypothesis was experimentally tested by restoring the standard consensus in the RH of *R. eutropha* H16.

Preliminary RH assays with soluble extracts showed that both the Ile/Val as well as the Phe/Leu exchange mutants were less stable in air while maintaining the activity under argon. This is the first evidence that the structure of the hydrophobic cavities may well affect the catalytic behaviour of a given hydrogenase.

**Signal transduction**

Three proteins, the response regulator HoxA, the histidine protein kinase HoxJ and the \([\text{NiFe}]\) hydrogenase RH are involved in the signal transduction cascade in *R. eutropha* H16. Mutants disrupted in the individual components gave first insights into the mechanism of signal transduction [31]. A mutant with a knockout in the central domain of HoxA has lost its ability to activate transcription of the MBH and SH genes as monitored with promoter fusions using the lacZ gene as a reporter. To our initial surprise an exchange of the putative phosphoryl receiver Asp-55 had just the opposite effect: the system was completely derepressed even in the absence of \(\text{H}_2\). Moreover, a mutation of the kinase motif in HoxJ resulted in a similar phenotype clearly indicating that unlike in most other two-component regulatory systems, phosphorylation of HoxA has a negative effect on transcription. A similar correlation has been observed for the
corresponding HupR/HupT couple in *R. capsulatus* [32]. Both, a functional input domain of HoxJ and an intact H$_2$-sensing protein obviously counteract the negative role of the kinase. If the input domain of HoxJ or the RH protein were mutated transcription was completely repressed, indicating that the H$_2$ signal could no longer be recognized [24].

To learn more about the interacting partners, the connection between the RH and the kinase HoxJ was studied in more detail. Mixing of purified components led to the formation of an RH–HoxJ complex that occurred with a high molecular mass in native PAGE. The complex maintained hydrogenase activity as visualized by an in-gel activity staining assay [32]. Complex formation is abolished, and the tetrameric RH dissociates into the dimeric conformation if a translational stop codon is inserted into the C-terminus of the small RH subunit gene *hoxB*. This mutation removes a peptide extension of 55 amino acids, a sequence that is well conserved in H$_2$-sensing proteins. The so-called RHStop mutant protein failed to sense the presence of H$_2$ to the cells, indicating that the C-terminal domain of HoxB is essential for signal transfer [33].

To facilitate the isolation of the RH–HoxJ complex, a single step purification method was developed using affinity chromatography. His-tagged kinase was immobilized on Ni$^{2+}$-nitrilotriacetate magnetic beads and incubated with RH-containing extracts. After washing the beads, the RH–HoxJ complex was eluted. The same method was also applied to analyse truncated mutant complexes. From these studies, we conclude that complex formation between the RH and the kinase HoxJ requires a complete hydrogenase module and only a short N-terminal peptide of the input domain of HoxJ that is missing even the PAS signature (O. Lenz and B. Friedrich, unpublished work). Determination of the size of the entire complex using gradient gels or gel filtration yielded a molecular mass of 350 and 380 kDa respectively (O. Lenz, T. Bührke and B. Friedrich, unpublished work). Thus a protein complex of approx. 350,000 Da consisting of two dimeric hydrogenase molecules and a tetrameric kinase is obviously required to detect H$_2$, the smallest molecule in nature.

To get further insights into the mechanism of H$_2$-guided signal transduction, preliminary phosphorylation assays were conducted by incubating the RH–HoxJ complex with [γ-32P]ATP. In the absence of H$_2$, two radioactively labelled bands were identified, the slowly migrating complex RH–HoxJ and a faster moving band representing the dissociated form of the kinase HoxJ. In a parallel experiment, in which H$_2$ was added to the assay, only a single phosphorylated signal correlating with the free HoxJ kinase was observed. The RH–HoxJ complex remained unphosphorylated. From these results, we conclude that phosphorylation of the complex is interrupted in the presence of H$_2$ (O. Lenz, A. Porthun and B. Friedrich, unpublished work). These observations are in agreement with the model of signal transduction presented in Figure 2.

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