Activation and sensing of hydrogen in nature
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The $\text{H}_2$ sensor of *Ralstonia eutropha*. Biochemical characteristics, spectroscopic properties, and its interaction with a histidine protein kinase

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The $H_2$ sensor of *R. eutropha*

**ABSTRACT**

Previous genetic studies have revealed a multicomponent signal transduction chain, consisting of a $H_2$ sensor, a histidine protein kinase and a response regulator, which controls hydrogenase gene transcription in the proteobacterium *Ralstonia eutropha*. In this study, we isolated the $H_2$ sensor and demonstrated that the purified protein forms a complex with the histidine protein kinase. Biochemical and spectroscopic analysis revealed that the $H_2$ sensor is a cytoplasmic [NiFe] hydrogenase with unique features. The $H_2$-oxidising activity was two orders of magnitude lower than that of standard hydrogenases and insensitive to oxygen, carbon monoxide and acetylene. Interestingly, only $H_2$ production but no HD formation was detected in the $D_2/H^+$ exchange assay. FTIR data showed an active site similar to that of standard [NiFe] hydrogenases. It is suggested that the protein environment accounts for a restricted gas diffusion and for the typical kinetic parameters of the $H_2$ sensor. EPR analysis demonstrated that the [4Fe-4S] clusters within the small subunit were not reduced under hydrogen even in the presence of dithionite. Optical spectra revealed the presence of a novel, redox-active, n=2 chromophore which is reduced by $H_2$. The possible involvement of this chromophore in signal transduction is discussed.
The detection of physiologically important gases by organisms is mediated by biological sensors which convert the molecular signal into a cellular response. Sensors for O₂, CO and NO have been described and the signalling mechanism is subject of current research (Ignarro, 1982; Gilles-Gonzalez, 1991; Shelver, 1997). One of the best-studied examples is the two-component FixL-FixJ system of *Rhizobium meliloti* and *Bradyrhizobium japonicum*. In this case the presence of O₂ is detected by a heme-containing histidine protein kinase (Gilles-Gonzalez, 1995). The heme group in FixL binds the oxygen molecule that induces a transition of the ferrous iron from high-spin to low-spin. This triggers the inactivation of the kinase domain of FixL. The release of O₂ at low O₂ tensions, restores the S = 2 state of the heme iron, which in turn leads to activation of the kinase by autophosphorylation. Subsequent phosphoryl transfer to the response activator FixJ finally stimulates gene transcription (Gong, 1998).

In the facultative chemolithotrophic proteobacterium *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*), the oxidation of molecular hydrogen is catalysed by two [NiFe] hydrogenases, a membrane-bound (MBH, Schink, 1979) and a cytoplasmic NAD⁺-reducing hydrogenase (SH, Schink, 1976). The structural genes of both [NiFe] hydrogenases together with sets of accessory genes are grouped in the MBH and SH operons, which are induced in the presence of molecular hydrogen (Lenz, 1998; Schwartz, 1998). Hydrogenase gene transcription is controlled by a multicomponent regulatory system consisting of the proteins HoxB, HoxC, HoxJ and HoxA, which are encoded in the MBH operon (Lenz, 1997; Lenz, 1998; Schwartz, 1998). HoxJ and HoxA share typical features of a bacterial two-component regulatory system that recognises and responds to various environmental stimuli (Stock, 1995; Lenz, 1998). Our studies showed that HoxJ displays autokinase activity (Lenz, 1998) and communicates with the activator HoxA (Förgber, M., Lenz, O., Schwartz, E. and Friedrich, B., unpublished results), a member of the NtrC family of response regulators (Eberz, 1991). HoxA, the final target of the H₂-sensing signal transduction chain, binds to the upstream region of the hydrogenase promoters and activates open complex formation by sigma 54 RNA polymerase (Zimmer, 1995; Schwartz, 1998).

Genetic studies revealed that recognition of H₂ requires in addition to HoxA and HoxJ the protein HoxBC (Lenz, 1998). Proteins similar to HoxBC, designated HupUV, have been identified in *Rhodobacter capsulatus* and *B. japonicum* (Elsen, 1996; Black, 1994). HoxBC-like proteins show typical features of a [NiFe] hydrogenase (Kleihues, 2000). Although HoxBC is essential for lithoautotrophic growth of *R. eutropha* (Lenz, 1998), it can not compensate for the loss of the MBH and the SH. This observation points to a regulatory rather than an energy-yielding function of the HoxBC protein (Kleihues, 2000). The low level of expression combined with an extremely low activity allowed only preliminary biochemical analysis of HoxBC in crude extracts (Pierik, 1998). Attempts to express a functional HoxBC protein in *Escherichia coli* were unsuccessful. This prompted us to develop a homologous overexpression system in *R. eutropha* (Kleihues, 2000) and to use it successfully for the purification of HoxBC, further on named regulatory hydrogenase (RH).
Biochemical and spectroscopic analysis of the homogenous RH uncovered unique enzymatic features, which are clearly distinct from the properties of standard hydrogenases. The data suggest that the RH shows a common [NiFe] active site but displays significant changes in the protein environment. In order to study the mechanism of H$_2$ signal transduction in more depth, we started to establish an in vitro system, using purified components. First data show that the RH forms a specific complex with the sensor kinase HoxJ, supporting the notion that the RH is a direct component of the signal transduction chain.

MATERIALS AND METHODS

Cell growth. R. eutropha strain HF371, a derivative of R. eutropha H16, harbouring plasmid pGE378, was used for protein purification (Kleihues, 2000). Cells were heterotrophically grown in a mineral medium in a 10 l Braun Biostat fermentor (Braun, Melsungen, Germany) at 30°C under hydrogenase derepressing conditions. At an optical density (OD$_{450}$) of 11 the cells were harvested, washed in 50 mM potassium phosphate buffer pH 7.0 (K-PO$_4$ buffer), and stored frozen in liquid nitrogen.

RH purification. Cells (83 g, wet weight) were resuspended in 30 ml of K-PO$_4$ buffer containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were disrupted by two passages through a chilled Amicon French press cell at 1100 psi (75.8 bar). Soluble extracts were prepared by high-speed centrifugation (100,000 × g, 60 min, 4°C). The resulting supernatant was degassed and saturated with hydrogen. The extract was kept under an atmosphere of 100% H$_2$ and subsequently incubated for 10 min at 65°C. After the heat treatment the sample was chilled on ice. All further purification steps were carried out under air. The denatured proteins were removed by centrifugation (13,000 × g, 20 min, 4°C) and the supernatant was fractionated by addition of (NH$_4$)$_2$SO$_4$ to a final concentration of 1 M. The precipitated proteins were removed by centrifugation (13,000 × g, 20 min, 4°C) and the clear supernatant was directly applied to a POROS 20ET column (Applied Biosystems; ethyl ether; 10 x 100 mm), pre-equilibrated with K-PO$_4$ buffer containing 1 M (NH$_4$)$_2$SO$_4$ at a flow rate of 40 ml/min (BioCAD Perfusion Chromatography Workstation). The column was washed with 2 bed volumes of K-PO$_4$ buffer containing 1 M (NH$_4$)$_2$SO$_4$. The protein was eluted with K-PO$_4$ buffer containing 0.4 M (NH$_4$)$_2$SO$_4$ and fractions of 4 ml were collected. The active fractions of several column runs were combined, concentrated and dialysed against K-PO$_4$ buffer. The RH was further purified on a POROS 20HQ column (Applied Biosystems; quaternised polyethyleneimine; 4.6 x 100 mm) pre-equilibrated with K-PO$_4$ buffer. The eluent was pooled, concentrated (Centriprep-10; Amicon) and directly frozen in liquid N$_2$. Protein concentrations were determined according to the method of Lowry, 1951.

Immunological procedures. Proteins were resolved by electrophoresis in 12% polyacrylamide/SDS gels and transferred to Protran BA85 nitrocellulose membranes (Schleicher & Schüll). HoxC was detected with anti-HoxC serum, diluted 1:1000, and an alkaline-phosphatase-labeled goat anti-rabbit IgG (Dianova, Hamburg).
Complex formation assay. The histidine protein kinase HoxJ was overproduced in *E. coli* and purified as a polyhistidine-tagged protein, His$_6$-HoxJ, by metal chelate affinity chromatography (Lenz, 1998). Purified His$_6$-HoxJ and RH were mixed and subsequently applied to a native 4-15% polyacrylamide gel. Native gel electrophoresis was carried out as previously described (Bernhard, 1996). Complex formation was either monitored by in-gel hydrogenase activity staining (Bernhard, 1996) or by protein staining using Coomassie blue.

Metal analysis. Nickel and iron were determined with a Hitachi 180-80 polarised Zeeman Atomic Absorption Spectrophotometer against a standard series. Samples were made devoid of extraneous metal ions by passage over a Chelex-100 column (BioRad).

Activity measurements. Hydrogen-uptake activity was measured amperometrically at 30°C in a cell (2.15 ml) with 50 mM Tris-HCl (pH 8.0) using a Clark-type electrode (YSI 5331) according to Coremans, 1989. As O$_2$ did not affect the activity, no efforts were made to remove air. Hydrogen, in the form of H$_2$-saturated water, was added to final concentrations varying from 36 μM to 100 μM. As electron acceptor either benzyl viologen (BV, 4.2 mM, $E_m$ = -359 mV) or methylene blue (MB, 4 mM, $E_m$ = +11 mV) were used. The measured specific activities were plotted against the H$_2$ concentration. The dependence was simulated using the program Leonora by Cornish-Bowden, assuming Michaelis-Menten kinetics (Cornish-Bowden, 1995). Protein concentrations in the assay were typically 2.5 to 5 nM RH ($\alpha$,$\beta$). Benzyl viologen-dependent H$_2$-evolution was determined amperometrically at 30°C. The reaction mixture contained 50 mM acetate buffer, 1 mM benzyl viologen and 3 mM sodium dithionite.

D$_2$/H$^+$ exchange activity was measured in a stirred membrane leak chamber fitted to a mass spectrometer (Masstor 200 DX quadrupole, VG Quadrupoles Ltd.). Two different assays were used. In the first assay 10 ml of MES / MOPS / Tris buffer solution (ionic strength 90 mM; pH 6.5) was saturated with 20% D$_2$ and 80% Ar and 1 μmol of sodium dithionite was added to eliminate residual oxygen. The reaction was started by the addition of RH ($\alpha$,$\beta$) to a final concentration of 0.12 μM. Masses 1-6 were scanned at 1 atomic mass unit per second. In the second assay the buffer solution was in 99.9% D$_2$O (Aldrich) and saturated with H$_2$. A control experiment was done in D$_2$/D$_2$O in order to evaluate the HD-production catalysed by the protein due to contaminant H$. This effect was subtracted from the H$_2$/D$_2$O assay. The pD of the assay mixtures was measured with a glass electrode calibrated with pH standards in H$_2$O. It was taken into account that pD = pH + 0.41 (Covington, 1968; Quinn, 1991). All experiments were done at 30°C.

EPR spectroscopy. X-band (9.4 GHz) spectra with a 100 kHz field-modulation frequency were recorded on a Bruker ECS106 EPR spectrometer equipped with an Oxford Instruments ESR900 helium-flow cryostat with an ITC4 temperature controller. The magnetic field was calibrated with an AEG magnetic field meter. The frequency was measured with a Hewlett Packard 5350B Microwave Frequency Counter. Illumination of the samples was performed by shining white light (Osram Halogen Bellaphot, 150 W) via a light guide through the irradiation grid of the Bruker ER 4102 ST cavity. Spectra were simulated according to previously published formulas (Beinert, 1982).
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**FTIR spectroscopy.** Fourier Transform Infrared (FTIR) spectra were taken on a BioRad FTS 60A spectrometer equipped with an MCT detector. Spectra were recorded at room temperature with a resolution of 2 cm$^{-1}$. Typically, averages of 1524 spectra were taken against proper blanks. Enzyme samples (10 µl) were loaded into a gas-tight transmission cell (CaF$_2$, 56 µm pathlength). The spectra were corrected for the baseline using a spline function provided by the BioRad software.

**Ultraviolet/visible spectroscopy.** Optical spectra were taken on an Aminco DW-2000 spectrophotometer interfaced with an IBM computer.

**RESULTS**

**Purification of the RH protein.** To avoid interferences with the dominant activities of the MBH and the SH we started the purification of the RH protein with mutant *R. eutropha* HF371, in which the MBH and SH genes had been deleted by mutation. After cell disruption and high speed centrifugation the soluble extract was incubated at 65°C for 10 min under an atmosphere of H$_2$ (100%). The heat-treatment was necessary prior to high-resolution hydrophobic interaction chromatography, to provide an effective and rapid isolation of the RH. Purification to apparent homogeneity was achieved by subsequent anion exchange chromatography. The total procedure is summarised in Table 1. Starting from 83 g of cells (wet weight) 10.9 mg of RH was obtained. The specific activity of the preparation was 0.94 U/mg of protein with MB as electron acceptor. The $H_2$-concentration in the assay was 57.8 µM. The protein was purified 26 fold with a yield of 6%.

**Table 1. Purification of the *R. eutropha* regulatory hydrogenase.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein mg</th>
<th>Total Activity units</th>
<th>Spec. Activity units/mg</th>
<th>Recovery %</th>
<th>Purification -fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble extract</td>
<td>4676</td>
<td>168.5</td>
<td>0.036</td>
<td>100</td>
<td>1.3</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>1734</td>
<td>83.3</td>
<td>0.048</td>
<td>49.4</td>
<td>1.4</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>1620</td>
<td>79.4</td>
<td>0.049</td>
<td>47.1</td>
<td>1.4</td>
</tr>
<tr>
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<td>23.4</td>
<td>0.673</td>
<td>13.9</td>
<td>18.7</td>
</tr>
<tr>
<td>HQ column</td>
<td>10.9</td>
<td>10.3</td>
<td>0.942</td>
<td>6.1</td>
<td>26.2</td>
</tr>
</tbody>
</table>

* The individual steps are described in detail under Materials and methods

**Biochemical properties.** Two protein bands occurred after denaturing the RH by SDS-PAGE corresponding to molecular masses of 37 and 55 kDa, respectively (Figure 1A). These values are in good agreement with those predicted from the nucleotide sequence of hoxB (36.5 kDa) and hoxC (52.4 kDa). The identity of the purified protein was confirmed by immunoblot analysis, using an antibody raised against the HoxC subunit of the RH (Figure 1B). Analysis of the enzyme on a Superdex G-200 (Amersham Pharmacia Biotech) revealed a single peak corresponding to a molecular mass of approximately 165 kDa (data not shown) indicating that the RH was purified as a tetramer
with a αβ2 structure. Atomic absorption spectroscopy (AAS) showed an average metal content of 11.2 Fe/Ni. After chelax treatment this ratio decreased to 7.6 Fe/Ni. The activity after the chelax-100 column was 75% of the initial activity.

The oxidation of H2 by the purified RH turned out to be O2 insensitive. The level of activity was the same in aerobic and anaerobic buffers. Moreover, the rate of H2 oxidation determined with MB as the electron acceptor did not show the typical lag phase found for most as-isolated [NiFe] hydrogenases. This observation is consistent with the result obtained with soluble extract (Pierik, 1998). The $K_m$ for H2 was 25 ± 5 μM and the calculated specific activity at $V_{max}$ conditions was 1.2 ± 0.2 U/mg of protein. The activity of the RH remained constant over a broad pH range between 5-10 irrespective of the used buffers (K-acetate, K-PO4 and Tris/HCl; 50 mM each), whereas most hydrogenases show a distinct pH optimum. In contrast to the H2-uptake activity, the production of H2 by the RH was pH-dependent. Highest H2 evolution rates (0.8 U/mg of protein) were obtained at pH 4.0 with benzyl viologen as electron donor. Acetylene has been shown to be a competitive inhibitor for several hydrogenases (Hyman, 1985; Zorin, 1996). Incubation of the RH with C2H2 did not affect the RH activity (data not shown).

Storage of the purified RH at 4°C under air or an atmosphere of 100% O2 resulted in a loss of 50% of the H2-dependent MB-reducing activity within 48 h. Replacement of the air atmosphere by 100% Ar or N2 caused a decrease of 20% of the activity within the same period of time. Addition of metal ions (Fe3+, Ni2+, Mn2+, Mg2+, Zn2+) or glycerol (20%) or addition of KCl up to 0.5 M did not affect the stability of the RH. The supply of dithionite or ferricyanide under anoxic conditions also did not contribute to the stability of the RH. Moreover, storage of the isolated RH under an atmosphere of 100% H2 inactivated the RH rapidly, 50% of its activity disappeared within 12 h. The H2 sensitivity contrasts data obtained with the soluble extract, which showed constant RH activity over a period of 24 h under comparable conditions. In all cases inactivation of the RH was irreversible.

Figure 1. Purification of the RH. (A) Coomassie blue staining and (B) Immunoblot analysis of protein samples from various purification steps after separation by SDS-PAGE. The subunits HoxB and HoxC of the RH are indicated by an arrow. Lane 1, soluble extract; lane 2, supernatant after treatment at 65°C for 10 min; lane 3, supernatant of 25% (NH4)2SO4 precipitation; lane 4, hydrophobic interaction; lane 5, anion exchange chromatography.
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**D'/H' exchange activity.** The D'/H' exchange assay with the RH yielded only H₃ production but no HD formation (Figure 2A). The initial rate of H₃ production at pH 6.5 was 2.1 ± 0.1 U/mg of protein. This behaviour is distinct from that of other [NiFe] hydrogenases, which show higher initial rates of HD-production than of H₃ production. When the exchange activity assay was measured in deuterated water saturated with H₂, some HD production was detected with the RH, although the rate of D₃ evolution was definitively higher (Figure 2B). The initial rate of D₃ production at pH 6.5 was 1.3 ± 0.2 U/mg of protein, whereas the initial rate of HD production was 0.5 ± 0.1 U/mg of protein. The pH optimum of the D'/H' exchange activity of the RH was at pH 5.5 (data not shown).

![Figure 2](image-url)

**Figure 2.** D'/H' exchange activity of the RH. (A) D₃/H' exchange kinetics catalysed by the RH from *R. eutropha* in Mes/Mops/Tris buffer solution (ionic strength 90 mM, pH 6.5) saturated with 20% D₂ and 80% Ar. (B) H₂/D' exchange kinetics measured in Mes/Mops/Tris buffer solution in D₂O (ionic strength 90 mM, pH 6.5) saturated with 100% H₂. The arrow marks the addition of the RH to a final concentration of 0.12 µM. Masses 2-4 were recorded.

**EPR spectroscopy.** Preliminary studies of the RH in crude cell extracts prohibited a study of the EPR properties of its Fe-S clusters (Pierik, 1998). The purified enzyme now enabled this approach. The as isolated RH showed no EPR signals at temperatures between 4.2 K and 100 K. Also after addition of the oxidising agent DCIP (dichlorophenolindophenol, E₅₀ = +230 mV) no signal occurred. Upon reduction of the RH (15 min under 100% H₂ at room temperature in 50 mM Tris-HCl, pH 8.0) a rhombic EPR signal with g values at 2.19, 2.13 and 2.01 appeared (Figure 3, trace A). The double-integrated intensity of the signal amounted to a spin concentration equal to 69% of the Ni concentration. The EPR signal is very similar to the well-studied Ni₄-C' signal observed in standard hydrogenases (e.g. from *Desulfovibrio gigas* and *Allochromatium vinosum*) and it is due to a paramagnetic state of the active site Ni in the 3+ state (Happe, 1999). A typical feature of enzyme in the Ni₄-C' state is its light sensitivity at cryogenic temperatures, yielding the so-called Ni₄-L' signal as a result of the photodissociation of a hydrogen (Van der Zwaan, 1985). A model for this photodissociation has been described in Happe, 1999. In the case of the RH the Ni₄-C' signal also showed this light-sensitive behaviour. Upon illumination at 30 K a spectrum (Ni₄-L': g₁ = 2.045, 2.09, 2.24), only slightly different from the Ni₄-L' signal of standard hydrogenases, showed up (Figure 3, trace B). The small difference concerns the position of the gₓ (2.24 in the RH as compared to 2.28/2.30...
in standard [NiFe] hydrogenases). This points to a small structural difference around the active-site nickel. Upon warming of the sample to 200 K for 15 min in the dark a third, transient, spectrum came up with g values at 2.047, 2.069 and 2.30 (Figure 3, trace C). Only after several hours at 200 K the sample returned to the Ni\textsubscript{L-C'} state. Contrary to observations in standard [NiFe] hydrogenases no signal of a [3Fe-4S]\textsuperscript{1+} cluster could be observed in the oxidised protein, not even after treatment with excess DCIP. This is in agreement with the presence of three [4Fe-4S] clusters as predicted from the amino-acid sequence data. When the protein was treated with 100\% H\textsubscript{2}, however, no signals due to reduced cubanes were detectable, not even if 20 mM dithionite was added.

None of the Ni signals (Ni\textsubscript{L-C'}, Ni\textsubscript{L-L'} or the transient signal) showed any spin coupling due to a reduced proximal [4Fe-4S] cluster (Figure 3). This indicates that this cluster was in the oxidised, diamagnetic state in the RH under H\textsubscript{2}. In standard [NiFe] hydrogenases the proximal cluster is usually reduced under 100\% H\textsubscript{2}. The interaction of the Ni with the reduced proximal cluster is observed as a clear two-fold splitting of the Ni\textsubscript{L-C'} signal at 4.5 K. At low temperatures it was also possible to completely saturate the Ni\textsubscript{L-C'} signal at high microwave power (260 mW) which is again indicative of an oxidised proximal cluster (Van der Zwaan, 1985; Teixeira, 1987). Reduction of the RH with dithionite in the presence or absence of low potential electron acceptors (MV, BV) under 100\% H\textsubscript{2} did not evoke any signal of a reduced Fe-S cluster. Also inspection of the integrated EPR signals did not uncover any broad signal due to reduced Fe-S clusters as can be seen in the right-hand panel in Figure 3 for the Ni\textsubscript{L-L'} signal.

Figure 3. EPR spectra at 4.5 K of the RH under 100\% H\textsubscript{2}. Left-hand panel: (A) The Ni\textsubscript{L-C'} state. (B) After illumination at 30 K (Ni\textsubscript{L-L'} state). (C) After 15 min at 200 K in the dark a transient state was observed. Relative gains for A, B and C are 1.6, 1 and 1. Right-hand panel: the light-induced Ni\textsubscript{L-L'} state is shown over a much wider field sweep (400 mT), either as the direct spectrum (E) or as the first integral (D). Trace D shows that there are no other paramagnets with broad signals hidden in the baseline of the first derivative. All spectra were recorded with a microwave power incident to the cavity of 0.26 \mu W.
**FTIR spectroscopy.** The FTIR measurements on purified RH confirmed the presence of only two redox states described earlier to be present in the RH from soluble extracts (Pierik, 1998). Untreated protein showed a spectrum (Figure 4A) with two small bands (2082 and 2071 cm⁻¹) and one large band (1943 cm⁻¹) in the 2150-1850 cm⁻¹ spectral region. This EPR-silent state of the active RH resembles the Ni₅-S state of standard [NiFe] hydrogenases. Maximal reduction, already obtained after a few minutes under 100% H₂ at room temperature, yielded the Ni₅-C⁻ state (Figure 4C) as identified previously in other [NiFe] hydrogenases (Bagley, 1995; DeLacey, 1997). This state showed a CO stretch vibration at 1960 cm⁻¹. The two bands at 2082 and 2071 cm⁻¹, which did not shift, are ascribed to the symmetrical and antisymmetrical coupled vibrations of two cyanides bound to Fe in the active site (Pierik, 1998). It was not possible to further reduce this state by adding excess dithionite (20 mM, spectrum not shown). When the gas phase was changed from 100% H₂ to 100% CO (equilibration time 60 min) a mixture of the Ni₅-C⁻ and Ni₅-S state was observed (Figure 4B). The spectrum clearly showed that it was not possible for exogenous CO to bind to the active site of the RH since no extra peak around 2060 cm⁻¹ could be seen. Such a band from added CO is observed in the A. vinosum and D. gigas enzyme (Bagley, 1994; A.L. DeLacey and V.M. Fernandez, unpublished result). A similar change was observed by replacing H₂ with Ar (results not shown). Upon complete oxidation with excess DCIP (2 mM) the sample returned to the Ni₅-S state.

![FTIR spectra of the RH. Trace A shows the RH in the oxidised state. After reduction under H₂, the RH ends up in the reduced state (trace C). If the gas phase was then exchanged for CO, a mixture of oxidised and reduced RH was observed (trace B). A similar spectrum was obtained by flushing with Ar.](image)

**UV-VIS absorption spectroscopy.** UV-VIS spectra of oxidised and reduced RH showed differences in absorption between the two species. Incubation of the RH under 100% H₂ resulted in an increase in absorption in the 250-280 nm and 300-400 nm spectral regions (Figure 5). The difference spectrum of reduced minus oxidised RH showed a large peak at 251 nm and a smaller one at 342 nm with an apparent shoulder at 305 nm. The calculated ε₂₅₁ was 11.96 mM⁻¹·cm⁻¹ based on protein concentration. Similarly the ε₃₄₂ was calculated to be 5.36 mM⁻¹·cm⁻¹. The protein concentration used (0.64 mg/ml) was such that the absorption at 280 nm was about 1.0. At this intensity the detector is still sensitive enough to pick up reliable differences in the UV, meaning that these are not due to mismatching in this region.
Complex formation. To elucidate the nature of the interaction between the RH and the signal transduction chain, purified kinase HoxJ and the RH were mixed and the sample was subjected to native PAGE (Figure 6). In one experiment the gel was resolved by protein staining (Figure 6A) and in the parallel experiment a hydrogenase in-gel activity staining with PMS as electron acceptor was performed (Figure 6B). Incubation of the RH with increasing amounts of HoxJ led to a band shift indicating the formation of a high molecular weight complex (Figure 6A, lanes 2-4). A band shift was not observed in the control containing the RH and an excess of bovine serum albumin (Figure 6A and B, lane 5). The in-gel assay (Figure 6B) demonstrated that the hydrogenase activity of the RH was maintained at high level upon complex formation (Figure 6B, lanes 3 and 4). Exposure to H₂ resulted in considerable loss of hydrogenase activity (Figure 6B, lane 6), which is consistent with the observed instability of the RH in the presence of H₂.

Figure 5. Difference UV-Vis spectrum of reduced minus oxidised RH. Aerobic RH was diluted to a concentration of 0.64 mg/ml and divided over two cuvets. One cuvet was put under 100% H₂ and measured against the aerobic sample in the reference cuvet at 2 nm resolution.

Figure 6. Complex formation of the RH and the histidine protein kinase HoxJ. Purified RH, HoxJ and a mixture of RH and HoxJ, pre-incubated for 10 min, were applied to native PAGE. (A) Coomassie staining and (B) H₂-dependent PMS reduction of native gels. Lane 1, 50 pmol RH; lane 2, 50 pmol RH and 10 pmol HoxJ; lane 3, 50 pmol RH and 50 pmol HoxJ; lane 4, 50 pmol RH and 250 pmol HoxJ; lane 5, 50 pmol RH and 250 pmol BSA; lane 6, 50 pmol RH and 50 pmol HoxJ pre-incubated for 30 min under H₂; lane 7, 50 pmol of HoxJ.
DISCUSSION

Genetic and biochemical studies uncovered a signal transduction chain, which directs H₂-dependent gene activation in *R. eutropha*. This signal transduction chain consists of the transcription activator HoxA, the histidine protein kinase HoxJ and the H₂-sensor RH. The RH is absolutely necessary for the recognition of dihydrogen suggesting its primary role in signal reception (Lenz, 1998). Sequence alignment revealed that the RH contains typical signatures of [NiFe] hydrogenases (Kleihues, 2000) and a preliminary EPR and FTIR study showed an active site similar to that of prototypic [NiFe] hydrogenases (Pierik, 1998). Characterisation of the purified RH achieved in this study confirmed some biochemical features which are compatible to those of standard [NiFe] hydrogenases. On the other hand, some characteristics were uncovered which are obviously uniquely assigned to the subgroup of H₂-sensing proteins (Kleihues, 2000). Unlike standard [NiFe] hydrogenases, which usually have H₂-uptake activities of about 200-300 U/mg of protein, the RH displayed a specific activity at V_{max} of only 1.2 ± 0.2 U/mg of protein. Moreover, in the air-oxidised state the RH showed no lag phase suggesting that it does not require a reductive activation step before the protein is enzymatically active.

Interestingly, the activity of the RH was not inhibited by O₂, CO or C₂H₂. Most hydrogenases are sensitive to these gases with the exception of the SH of *R. eutropha* (Schneider, 1979). In this case a modified catalytic centre probably excludes the binding of CO and O₂ (Happe, 2000). Although the EPR and FTIR spectra of the Ni-Fe site of the RH resemble those of standard [NiFe] hydrogenases, the active site of the RH exhibits some important redox differences. Only the Ni₁-S and Ni₁-C₁ states are attainable and CO cannot bind to the active enzyme. This indicates that the Ni site (where in standard [NiFe] hydrogenases CO binds and where H₂ is proposed to react under turnover conditions, Happe, 1999) is altered such that it cannot react with CO or H₂. This would restrict the reaction with H₂ to the Fe site resulting in the Ni₁-C₁ state only. The very low activity of the RH is in line with this idea. The D₂/H⁺ exchange data suggest that D₂ diffusion to and from the active site is severely restricted resulting in a molecular cage effect (Krasna, 1979). The formed HD then reacts again to form H₂, before diffusion of HD from the enzyme to the bulk occurs. In the H₂/D₂ exchange, the formed D₂ escapes slower than HD, allowing some HD detection. The gas channel detected in the X-ray structures of [NiFe] hydrogenases (Volbeda, 1995; Montet, 1997) points right to the Ni site. Changes in the amino-acid composition of this channel close to the Ni site, e.g. the presence of more bulky residues, could explain both the redox and the exchange properties. The kinetic behaviour of the RH in the D₂/H⁺ activity assay is in agreement with the low activity of the RH in the other assays.

The described EPR and FTIR data on the purified RH do not differ from those presented earlier for the protein in crude extracts, so purification does not change these properties. A previously unobserved state occurred when the Ni₁-L⁻ state was warmed up to 200 K. A transient state was then observed with g values at 2.047, 2.069 and 2.30. This points to changes induced in the vicinity of the Ni site. As yet, we do not understand the nature of these changes.

Another typical feature of the light sensitivity in the RH is that all conversions are much slower than in several other hydrogenases tested in this laboratory using the same experimental set-up (e.g. *A. vinosum, Methanococcus thermoautotrophicum, Wollinella succinogenes*). The Ni₁-C₁ to Ni₁-L⁻
conversion in membrane-bound hydrogenase (MBH) of *A. vinosum* is completed within 5 min whereas in the RH it took about 15 min. The difference in the reverse reaction was even more pronounced. After two hours at 200 K the RH was still in the transient dark state, whereas the MBH of *A. vinosum* requires only 10 to 15 minutes at 200 K to return completely to the Ni$_{2}$-C'. This slow photolysis and the extremely slow annealing might be due to a less spacious, obstructed active site. It was shown that it was impossible to reduce the three [4Fe-4S] clusters (predicted to be present from sequence data), although highly reductive conditions were applied (100% H$_{2}$ with benzyl viologen, methyl viologen and/or 20 mM dithionite). Also no splitting of the Ni$_{2}$-C' or Ni$_{2}$-L' signals at 4.5 K by a reduced proximal cluster was observed.

Our current model of signal transduction in the RH is as follows: H$_{2}$ binds to the active site (presumably at the Fe site, Happe, 1999) and causes a formal oxidation of the Ni ion from the 2+ to the 3+ state. The released electron is transferred to the Fe-S clusters. However no spectroscopic evidence for a reduced Fe-S cluster was found and no other S = 1/2 EPR signal was detected. Since the RH is apparently functional as an αβ tetramer the possibility exists that two unpaired spins released by the two Ni-Fe sites in the tetramer are united in a yet undetected, diamagnetic prosthetic group. Hence, UV-VIS spectroscopy was applied. Much to our surprise reduction of the RH by H$_{2}$ resulted in an increase in absorption with clear maxima at 251 and 342 nm. We tentatively conclude that this increase is caused by the reduction of a two electron accepting cofactor, shared by the two dimer (αβ) molecules in the RH (αβ). The exact identity of this cofactor is currently under investigation. The position of the 342 nm band and its approximate molecular absorption coefficient (5.4 mM$^{-1}$cm$^{-1}$) resemble those of NADH.

Transmission of the H$_{2}$-induced changes in the RH to the histidine protein kinase HoxJ proceeds via direct protein-protein interaction as shown by complex formation. The N-terminal part of HoxJ, the so-called input domain, is the most likely region for the signal-accepting site. Sequence comparison revealed that this domain is a member of the PAS domain superfamily, which is found in a wide variety of regulatory systems involved in the sensing of light, oxygen or redox potential (Ponting, 1997; Zhulin, 1997; Taylor, 1999). Several PAS domain proteins mediate signal transmission by the way of an associated cofactor (Taylor, 1999), like the FAD in the aerotaxis signal transducer Aer of E. coli (Bibikov, 2000). Such a two-electron cofactor in HoxJ might be a good candidate and might be reduced by the yet unidentified cofactor in the RH. In this scenario, electron flow from the RH to the histidine kinase should induce a conformational switch to modulate the activity of the HoxJ transmitter domain and thereby affecting the autophosphorylation activity of HoxJ. To resolve such a mechanism we intend to block electron transport within the RH by site directed mutagenesis. Attractive targets will be the ligands of the three Fe-S clusters and the non-metal cofactor of the RH.

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The $H_2$ sensor of R. eutropha