The $\text{H}_2$ Sensor of \textit{Ralstonia eutropha}

\textbf{BIOCHEMICAL CHARACTERISTICS, SPECTROSCOPIC PROPERTIES, AND ITS INTERACTION WITH A HISTIDINE PROTEIN KINASE}$^*$

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Previous genetic studies have revealed a multicomponent signal transduction chain, consisting of an $\text{H}_2$ sensor, a histidine protein kinase, and a response regulator, which controls hydrogenase gene transcription in the proteobacterium \textit{Ralstonia eutropha}. In this study, we isolated the $\text{H}_2$ sensor and demonstrated that the purified protein forms a complex with the histidine protein kinase. Biochemical and spectroscopic analysis revealed that the $\text{H}_2$ sensor is a cytoplasmic [NiFe]-hydrogenase with unique features. The $\text{H}_2$-oxidizing activity was $2$ orders of magnitude lower than that of standard hydrogenases and insensitive to oxygen, carbon monoxide, and acetylene. Interestingly, only $\text{H}_2$ production but no HD formation was detected in the D$_2$/H$_2$ exchange assay. Fourier transform infrared 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 $\text{H}_2$ 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 that is reduced by $\text{H}_2$. The possible involvement of this chromophore in signal transduction is discussed.

The detection of physiologically important gases by organisms is mediated by biological sensors that convert the molecular signal into a cellular response. Sensors for $\text{O}_2$, $\text{CO}$, and $\text{NO}$ have been described, and the signaling mechanism is the subject of current research (1–3). One of the best studied examples is the two-component FixL-FixJ system of \textit{Rhizobium meliloti}. In this case, the presence of $\text{O}_2$ regulates the expression of genes involved in nitrogen fixation. The $\text{H}_2$ sensor is a novel, redox-active, $n = 2$ chromophore that is reduced by $\text{H}_2$. The possible involvement of this chromophore in signal transduction is discussed.

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1 The abbreviations used are: MBH, membrane-bound hydrogenase; DCIP, dichlorophenolindophenol; PETR, Fourier transform infrared; RH, regulatory hydrogenase; SH, soluble hydrogenase; PAGE, polyacrylamide gel electrophoresis; Mes, $4$-morpholinolinethanesulfonic acid; Mops, $4$-morpholinepropanesulfonic acid.
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 H2 signal transduction in more depth, we began 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.

**EXPERIMENTAL PROCEDURES**

**Cell Growth—**R. eutropha strain HF371, a derivative of R. eutropha H16, harboring plasmid pGE378, was used for protein purification (16). Cells were heterotrophically grown in a mineral medium in a 10-liter Braun Biostat fermentor (Braun, Melsungen, Germany) at 30 °C under hydrogenase derepressing conditions. At an OD660 of 11 the cells were harvested, washed in 50 mM potassium phosphate buffer, pH 7.0 (K-PO4 buffer), and stored frozen in liquid nitrogen.

**RH Purification—**Cells (83 g, wet weight) were resuspended in 30 ml of K-PO4 buffer containing 0.1 mM phenylmethylsulfonyl fluoride. Cells were disrupted by two passages through a chilled Amicon French press cell at 1100 pounds/square inch (75.8 bar). The denatured proteins were removed by centrifugation (13 000 × g, 20 min, 4 °C), and the supernatant was fractionated by addition of (NH4)2SO4 to give 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 20E column (Applied Biosystems; ethyl ether; 10 × 100 mm). Equilibration was achieved by subsequent anion exchange chromatography to provide a final solution of 1 M. The protein was eluted with K-PO4 buffer containing 0.4 M (NH4)2SO4, and fractions of 4 ml were collected. The active fractions of several column runs were pooled, concentrated, and dialyzed against K-PO4 buffer. The RH was further purified on a POROS 20HQ column (Applied Biosystems; quarterized polyethyleneimine; 4.6 × 100 mm) equilibrated with K-PO4 buffer. The eluent was pooled, concentrated (Centriprep-10; Amicon), and directly frozen in liquid N2. Protein concentrations were determined according to the methods of Lowry et al. (18).

**Immunological Procedures—**Proteins were resolved by electrophoresis in 12% polyacrylamide/SDS gels and transferred to Protran BA85 nitrocellulose membranes (Schleicher & Schuell). HoxC was detected with anti-HoxC serum, diluted 1:1000, and an alkaline-phosphatase–nitrocellulose membranes (Schleicher & Schuell). HoxC was detected using a standard series. Samples were made devoid of extraneous metal ions by passage over a Chelex-100 column (Bio-Rad).

**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 et al. (20). As O2 did not affect the activity, no efforts were made to remove air. Hydrogen, in the form of H2-saturated water, was added to final concentrations varying from 36 to 100 μM. As electron acceptor either benzyl viologen (4.2 mM, E1/2 = −359 mV) or methylene blue (4 mM, E1/2 = +11 mV) were used. The measured specific activities were plotted against the H2 concentration. The dependence was simulated using the program Leonora by Cornish-Bowden, assuming Michaelis-Menten kinetics (21). Protein concentrations in the assay were typically 2.5–5 μM RH (αβ). Benzyl viologen-dependent H2 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/H exchange activity was measured in a stirred membrane leak chamber fitted to a mass spectrometer (Masstorr 200 DX quadrupole, VG Quadrupoles Ltd.). Two different assays were used. In the first assay 10 ml of Mes/Mop/Tris buffer solution (ionic strength 90 mM; pH 6.5) was saturated with 20% D2 and 80% argon, and 1 μM of sodium dithionite was added to eliminate residual oxygen. The reaction was started by the addition to the buffer by a final concentration of 1.2 μM. Masses 1–6 were scanned at 1 atomic mass unit/s. In the second assay the buffer solution was in 99.9% D2O (Aldrich) and saturated with H2. A control experiment was done in D2/O in order to evaluate the HD production catalyzed by the protein due to contaminant H+. This effect was subtracted to the H2/D2O assay. The pD of the assay mixtures was measured with a glass electrode calibrated with pH standards in H2O.

**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 supernatant was incubated at pH 5.5 of 100 μM. As electron acceptor either benzyl viologen (4.2 mM, E1/2 = −359 mV) or methylene blue (4 mM, E1/2 = +11 mV) were used. The measured specific activities were plotted against the H2 concentration. The dependence was simulated using the program Leonora by Cornish-Bowden, assuming Michaelis-Menten kinetics (21). Protein concentrations in the assay were typically 2.5–5 μM RH (αβ). Benzyl viologen-dependent H2 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/H exchange activity was measured in a stirred membrane leak chamber fitted to a mass spectrometer (Masstorr 200 DX quadrupole, VG Quadrupoles Ltd.). Two different assays were used. In the first assay 10 ml of Mes/Mop/Tris buffer solution (ionic strength 90 mM; pH 6.5) was saturated with 20% D2 and 80% argon, and 1 μM of sodium dithionite was added to eliminate residual oxygen. The reaction was started by the addition to the buffer by a final concentration of 1.2 μM. Masses 1–6 were scanned at 1 atomic mass unit/s. In the second assay the buffer solution was in 99.9% D2O (Aldrich) and saturated with H2. A control experiment was done in D2/O in order to evaluate the HD production catalyzed by the protein due to contaminant H+. This effect was subtracted to the H2/D2O assay. The pD of the assay mixtures was measured with a glass electrode calibrated with pH standards in H2O.

**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 (Osmar Halogen Bellapohit, 150 watts) via a light guide through the irradiation grid of the Bruker ER 4102 ST cavity. Spectra were simulated according to the formulas published by Beinert and Albracht (24). **FTIR Spectroscopy—**Fourier transform infrared (FTIR) spectra were taken on a Bio-Rad 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 after 100 scans. Enzyme samples (10 μl) were loaded into a gas-tight transmission cell (CaF2, 56 μm path length). The spectra were corrected for the base line using a spline function provided by the Bio-Rad software. **Ultraviolet/Visible Spectroscopy—**Optical spectra were taken on an Amino DW-2000 spectrophotometer interfaced with an IBM computer.

### Table I: Purification of the R. eutropha regulatory hydrogenase

| Step               | Total protein | Total activity | Specific activity | Recovery | Purification |
|--------------------|---------------|----------------|-------------------|----------|--------------|
| Soluble extract    | 4676          | 168.5          | 0.036             | 100      | 1.3          |
| Heat treatment     | 1734          | 83.3           | 0.048             | 49.4     | 1.3          |
| (NH4)2SO4          | 1620          | 79.4           | 0.049             | 47.1     | 1.4          |
| ET column          | 34.8          | 23.4           | 0.673             | 13.9     | 18.7         |
| HQ column          | 10.9          | 10.3           | 0.942             | 6.1      | 26.2         |

*The individual steps are described in detail under “Experimental Procedures.”*
Biochemical Properties—Two protein bands occurred after denaturing the RH by SDS-PAGE corresponding to molecular masses of 37 and 55 kDa, respectively (Fig. 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 (Fig. 1B). Analysis of the enzyme on a Superdex G-200 (Amersham Pharmacia Biotech) revealed a single peak corresponding to a molecular mass of ~165 kDa (data not shown) indicating that the RH was purified as a tetramer with an a2b2 structure. Atomic absorption spectroscopy showed an average metal content of 11.2 iron/nickel. After Chelex treatment this ratio decreased to 7.6 iron/nickel. The activity after the Chelex-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 methylene blue as the electron acceptor did not show the typical lag phase that is found with most isolated [NiFe]-hydrogenases. This observation is consistent with the result obtained with soluble extract (17). The Km for H2 was 25 ± 5 μM, and the calculated specific activity at Vmax conditions was 1.2 ± 0.2 units/mg of protein. The activity of the RH remained constant over a broad pH range between 5 and 10 irrespective of the used buffers (potassium 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 units/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 (25, 26). 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 methylene blue-reducing activity within 48 h. Replacement of the air atmosphere by 100% argon or N2 caused a decrease of 20% of the activity within the same period. Addition of metal ions (Fe3+, Ni2+, Mn2+, Mg2+, and Zn2+) or addition of KCl up to 0.5 M did not affect the stability of the RH. The supply of dithionite or ferriyianide under anaerobic 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 with 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.

D+/H+ Exchange Activity—The D+/H+ exchange assay with the RH yielded only H2 production but no HD formation (Fig. 2A). The initial rate of H2 production at pH 6.5 was 2.1 ± 0.1 units/mg of protein. This behavior is distinct from that of other [NiFe]-hydrogenases, which show higher initial rates of HD production than of H2 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 D2 evolution was definitively higher (Fig. 2B). The initial rate of D2 production at pH 6.5 was 1.3 ± 0.2 units/mg of protein, whereas the initial rate of HD production was 0.5 ± 0.1 units/mg of protein. The pH optimum of the D+/H+ exchange activity of the RH was at pH 5.5 (data not shown).

EPR Spectroscopy—Preliminary studies of the RH in crude cell extracts prohibited a study of the EPR properties of its Fe-S clusters (17). The purified enzyme now allowed this approach. The as isolated RH showed no EPR signals at temperatures between 4.2 and 100 K. Also after addition of the oxidizing agent DCIP (dichloro-phenolindophenol, E = +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 (Fig. 3, trace A). The double-integrated intensity of the signal amounted to a spin concentration equal to 69% of the nickel concentration.

The EPR signal is very similar to the well studied Ni5-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 nickel in the 3+ state (27). A typical feature of enzyme in the Ni5-C* state is its light sensitivity at cryogenic temperatures, yielding the so-called Ni5-L* signal as a result of the photodissociation of a hydrogen (28). A model for this photodissociation has been described by Happe et al. (27).

In the case of the RH the Ni5-C* signal also showed this light-sensitive behavior. Upon illumination at 30 K a spectrum (Ni5-L*: gmax = 2.045, 2.09, and 2.24), only slightly different from the Ni5-L* signal of standard hydrogenases, appeared (Fig. 3, trace B). The small difference concerns the position of the g1 (2.24 in the RH as compared with 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 (Fig. 3, trace C). Only after several hours at 200 K the sample returned to the Ni5-C* state.

Contrary to observations in standard [NiFe]-hydrogenases no signal of a [3Fe-4S] cluster could be observed in the oxidized 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% H2, however, no signals due to reduced cubanes were detectable, not even if 20 mM dithionite was added. None of the nickel signals (Ni5-C*, Ni5-L*, or the transient signal) showed any spin coupling due to a reduced proximal [4Fe-4S] cluster (27). This indicates that this cluster was in the oxidized, diamagnetic state in the RH under H2. In standard [NiFe]-hydrogenases the proximal cluster is usually reduced under 100% H2. The interaction of the nickel with the reduced proximal cluster is observed as a clear 2-fold splitting of the Ni5-C* signal at 4.5 K. At low temperatures it was also possible to completely saturate the Ni5-C* signal at high microwave power (260 milliwatts), which is again indicative of an oxidized proximal cluster (28, 29). Reduction of the RH with

![Fig. 1. Purification of the RH. A, Coomassie Blue staining; 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.](http://www.jbc.org/content/15594/1/15594/F1)
Dithionite in the presence or absence of low potential electron acceptors (methyl viologen and benzyl viologen) under 100% H₂ 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 Fig. 3 for the Ni a-L* signal.

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 (17). Untreated protein showed a spectrum (Fig. 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 Ni a-S state of standard [NiFe]-hydrogenases. Maximal reduction, already obtained after a few minutes under 100% H₂ at room temperature, yielded the Ni a-C* state (Fig. 4C) as identified previously in other [NiFe]-hydrogenases (30, 31). 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 anti-symmetrical coupled vibrations of two cyanides bound to iron in the active site (17). It was not possible to reduce further 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 Ni a-C* and Ni a-S state was observed (Fig. 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 (32). A similar change was observed by replacing H₂ with argon (results not shown). Upon complete oxidation with excess DCIP (2 mM) the sample returned to the Ni a-S state.

UV-Visible Absorption Spectroscopy—UV-visible spectra of oxidized 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 and 300–400 nm spectral regions (Fig. 5). The difference spectrum of reduced minus oxidized 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

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3 De Lacey, A.L., and Fernandez, V.M., unpublished results.
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 (Fig. 6). In one experiment the gel was resolved by protein staining (Fig. 6A), and in the parallel experiment a hydrogenase in-gel activity staining with phenazine methosulfate as electron acceptor was performed (Fig. 6B). Incubation of the RH with increasing amounts of HoxJ led to a bandshift indicating the formation of a high molecular weight complex (Fig. 6A, lanes 2–4). A bandshift was not observed in the control containing the RH and an excess of bovine serum albumin (Fig. 6, A and B, lane 5). The in-gel assay (Fig. 6B) demonstrated that the hydrogenase activity of the RH was maintained at high level upon complex formation (Fig. 6B, lanes 3 and 4). Exposure to H₂ resulted in considerable loss of hydrogenase activity (Fig. 6B, lane 6), which is consistent with the observed instability of the RH in the presence of H₂.

**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 (9). Sequence alignment revealed that the RH contains typical signatures of [NiFe]-hydrogenases (16), and a preliminary EPR and FTIR study showed an active site similar to that of prototypic [NiFe]-hydrogenases (17). Characterization of the purified RH achieved in this study confirmed some biochemical features that are compatible to those of standard [NiFe]-hydrogenases. On the other hand, some characteristics were uncovered that are obviously uniquely assigned to the subgroup of H₂-sensing proteins (16). Unlike standard [NiFe]-hydrogenases, which usually have H₂ uptake activities of about 200–300 units/mg of protein, the RH displayed a specific activity at 0.2 units/mg protein. Moreover, in the air-oxidized 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* (33). In this case a modified catalytic center probably excludes the binding of CO and O₂ (34). Although the EPR and FTIR spectra of the [NiFe] site of the RH resemble those of standard [NiFe]-hydrogenases, the active site of the RH exhibits some important redox differences. Only the Ni₅-S and Ni₃-C⁺ states are attainable, and CO cannot bind to the active enzyme. This indicates that the nickel site (where in standard [NiFe]-hydrogenases CO binds and where H₂ is proposed to react under turnover conditions (27)) is altered such that it cannot react with CO or H₂. This would restrict the reaction with H₂ to the iron site resulting in the Ni₃-C⁺ 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 (35). The formed HD then reacts again to form H₂, before diffusion of HD from the enzyme to the bulk occurs. In the H₂/D⁺ exchange, the formed D₂ escapes slower

![FIG. 4. FTIR spectra of the RH. Trace A shows the RH in the oxidized 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 oxidized and reduced RH was observed (trace B). A similar spectrum was obtained by flushing with argon.](image)

![FIG. 5. Difference spectrum of reduced minus oxidized RH.](image)

![FIG. 6. Complex formation of the RH and the histidine protein kinase HoxJ. Purified RH, HoxJ, and a mixture of RH and HoxJ, preincubated for 10 min, were applied to native PAGE. A, Coomassie staining; B, H₂-dependent PMS reduction of native gels. Lane 1, 50 pmol of RH; lane 2, 50 pmol of RH and 10 pmol of HoxJ; lane 3, 50 pmol of RH and 50 pmol of HoxJ; lane 4, 50 pmol of RH and 250 pmol of HoxJ; lane 5, 50 pmol of RH and 250 pmol of bovine serum albumin; lane 6, 50 pmol of RH and 50 pmol of HoxJ preincubated for 30 min under H₂; lane 7, 50 pmol of HoxJ.](image)
than HD allowing some HD detection. The gas channel detected in the x-ray structures of [NiFe]-hydrogenases (36, 37) points right to the nickel site. Changes in the amino acid composition of this channel close to the nickel site, e.g., the presence of more bulky residues, could explain both the redox and the exchange properties. The kinetic behavior of the RH in the $D_2/H^+$ 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 Ni₆-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 nickel 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 setup (e.g., Allochromatium vinosum, Methanococcus thermautotrophicum, and Wollinella succinogenes). The Ni₆-C* to Ni₆-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 2 h at 200 K the RH was still in the transient dark state, whereas the MBH of A. vinosum requires only 10–15 min at 200 K to return completely to the Ni₆-C* state. 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% H₂).

Detection of the H₂-induced changes in the RH by electron cofactor in HoxJ might be a good candidate to be 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 affect 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 Fe₅S clusters and the nonmetal cofactor of the RH.

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The H₂ Sensor of *Ralstonia eutropha*: BIOCHEMICAL CHARACTERISTICS, SPECTROSCOPIC PROPERTIES, AND ITS INTERACTION WITH A HISTIDINE PROTEIN KINASE

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