Characterization of the active site of a hydrogen sensor from *Alcaligenes eutrophus*

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Abstract A third hydrogenase was recently identified in the proteobacterium *Alcaligenes eutrophus* as a constituent of a novel H_2-sensing multicomponent regulatory system. This regulatory hydrogenase (RH) has been overexpressed in cells deficient in both the NAD^+-reducing [NiFe]-hydrogenase and the membrane-bound [NiFe]-hydrogenase. EPR, FTIR and activity studies of membrane-free extracts revealed that the RH has an active site much like that of standard [NiFe]-hydrogenases, i.e. a Ni-Fe site with two CN^- groups and one CO molecule. Its catalytic power is low, but the RH is always active, insensitive to oxygen, and occurs in only two redox states.

Key words: Hydrogen; Sensor; Active site; Hydrogenase; Electron paramagnetic resonance; Fourier transform infrared spectroscopy

1. Introduction

Hydrogenases are widespread among microorganisms and catalyze the activation of molecular hydrogen. Two classes of metal-containing hydrogenases are known: (A) enzymes possessing both nickel and iron ([NiFe]-hydrogenases (for review see [1]) and (B) enzymes which only contain iron ([Fe]-hydrogenases; for review see [2]). A third class of hydrogenases, not containing any metals and only active in the presence of its second substrate, has been described by Thauer and coworkers [3].

[NiFe]-hydrogenases contain two subunits which are homologous in all enzymes of this class. The first crystal structure of a [NiFe]-hydrogenase [4] revealed that four strictly conserved Cys residues in the largest subunit bind a bimetallic Ni-Fe center. Three non-protein ligands to Fe [4,5] have been characterized by FTIR studies as two non-exchangeable CN^- groups and one CO molecule [6]. The small subunits of [NiFe]-hydrogenases all display a conserved pattern, $CxxCx_nGxCxxxGx_mGCPP$ ($n = 61$–106, $m = 24$–61 [1]), binding one [4Fe-4S] cluster. This cluster is close to the active site [4] and is called the proximal cluster. In most, but not all, sequences six to eight additional cysteine residues are present, which harbor two more clusters, in the *Desulfovibrio gigas* enzyme being a second cubane cluster (distal) and a [3Fe-4S] cluster.

Biosynthesis of active [NiFe]-hydrogenases involves a series of post-translational steps including metal supply, assembly of the active site, proteolytic maturation [7,8] and in the case of membrane-bound and periplasmic hydrogenases Sec-independent protein export [9]. Especially in those organisms which harbor multiple hydrogenases with distinct physiological functions, these complex biosynthetic reactions need to be coordinated at the regulatory level.

The facultative chemolithoautotrophic proteobacterium *Alcaligenes eutrophus* contains two energy-generating [NiFe]-hydrogenases: (i) a membrane-bound enzyme (MBH), which is coupled to oxidative phosphorylation [10], shows the standard composition of [NiFe]-hydrogenases [11]; (ii) a cytoplasmic hydrogenase (SH) [12], capable of reducing NAD^+ and containing, in addition to the hydrogenase moiety, an iron flavoprotein [13,14]. Recently, a third hydrogenase was uncovered in *A. eutrophus* as a constituent of a regulatory H_2-sensing multicomponent system [15]. It is not involved in energy generation, but appears to be instrumental in H_2 recognition. Both the enzymatic and the sensing activity of this regulatory hydrogenase (RH) are dependent on Ni (Kleihues, Lenz, Bernhard and Friedrich, in preparation). Homologues of the RH with regulatory features, denoted HupUV, were also identified in *Bradyrhizobium japonicum* [16] and *Rhodobacter capsulatus* [17]. The latter can bind H_2 [18]. The primary structure of these H_2 sensor proteins reveals almost all conserved signatures for metal coordination in [NiFe]-hydrogenases. The small subunit of the regulatory hydrogenases, however, is devoid of an N-terminal leader sequence, pointing to a cytoplasmic location. Moreover, the large subunit lacks the C-terminal extension of standard [NiFe]-hydrogenases, the presence and removal of which are essential for the course of metal center assembly in these enzymes [19–22].

In this study we report on the first FTIR and EPR properties of a H_2-sensing protein, unveiling the identity of its active site.

2. Materials and methods

Plasmid pGE378 was used for RH overproduction in the strain *A. eutrophus* HF371, which harbors in-frame deletions in the structural genes of both MBH and SH [23]. For construction of pGE378 the RH-encoding genes *hoxB* and *hoxC* were fused to the strong promoter and translation initiation regions of the *A. eutrophus* soluble hydrogenase operon. The fusion was subsequently transferred into the broad-host-range vector pEDY309 to give plasmid pGE378. The detailed construction of pGE378 will be described elsewhere (Kleihues, Lenz, Bernhard and Friedrich, in preparation). *A. eutrophus* HF371, harboring the vector pEDY309, served as control and is defined as RH^-. *Alcaligenes* strains were grown heterotrophically at 30°C in a synthetic medium containing 0.2% (w/v) fructose, 0.2% (v/v) glycerol,

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Abbreviations: SH, soluble (cytoplasmic) hydrogenase; MBH, membrane-bound hydrogenase; RH, regulatory hydrogenase from *A. eutrophus*; RH^+, cells deficient in both the SH and the MBH, in which the RH was overproduced from the vector pGE378; RH^-, cells deficient in both the SH and the MBH, containing the vector control pEDY309

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and 1 μM $NiCl_2$ as described previously [24]. Tetracycline was supplemented at a concentration of 10 mg/l. After 50 h of growth (optical density at 436 nm of 10), cells were harvested by centrifugation (10 min at 5200×*g*) and washed once with 100 ml buffer (50 mM potassium phosphate buffer; pH 7.0). The yield was about 1.5 g (wet weight; 250 ml batch culture). The cells were resuspended in buffer (2 g/ml) and disrupted by one passage through a chilled 5 ml Amicon French Press cell at 900 psi (138 MPa) at a flow rate of 0.5 ml/min. Insoluble material was removed by centrifugation (10 min at 4300×*g*, followed by 60 min at 100 000×*g*). The clear supernatant was directly used for activity measurements (prior to freezing), or frozen in liquid nitrogen in 50 μl droplets (FTIR) or in EPR tubes, for later measurements. Protein concentrations were determined as described [25–27], using bovine serum albumin as standard.

Routine hydrogenase activities were measured as before [28]. To inspect the activation behavior of the enzyme, a solution of N_2-saturated 50 mM Tris-HCl buffer (pH 7), 10–25 mM benzyl viologen and 62 μM H_2 was used. The reaction was started by addition of enzyme solution. As described in Section 3, the use of air-saturated buffer did not change the behavior of the enzyme.

FTIR measurements were performed at room temperature on a Bio-Rad FTS 60A spectrometer equipped with an MCT detector. The spectra were recorded at a resolution of 2 cm^{-1}, and are averages of 1500–3000 scans. Membrane-free extracts (60–70 mg protein/ml) were loaded into a gas-tight IR transmittance cell with CaF_2 windows. The cell path length was 56 μm, maintained with a teflon spacer. A cell filled with extract from an RH^- strain was used as reference. For initial measurements and the extract from RH^- strains, the multipoint method of the Bio-Rad spectrometer was used for baseline corrections. In the case of reduced samples, the cell was flushed with Ar before loading.

EPR spectra (9 GHz) were obtained with a Bruker ECS-106 EPR spectrometer [29]. Illumination of the samples was performed by shining white light (Osram Halogen Bellaphot, 150 W) via a light guide into the Bruker ER 4102 ST cavity. Spectra were simulated and quantified as before [28].

3. Results

3.1. Activity and insensitivity toward O_2

Maximal H_2 uptake activity with RH^+ extracts (0.12 U/mg) was obtained with excess of benzyl viologen (25 mM). Storage in liquid nitrogen had no effect. In no case could a lag phase in the activity kinetics, as observed with the majority of [NiFe]-hydrogenases, be detected. It was discovered that the enzymatic properties did not alter when assayed in aerobic buffer. An example is provided in Fig. 1. The extract of RH^- cells contained only a barely detectable activity; these cells still contain trace amounts of the RH, equivalent to the level detected in wild-type cells. Addition of aerobically prepared extract of the RH^+ strain to the air-saturated buffer immediately resulted in uptake of hydrogen. From the trace a K_m value for H_2 of about 5 μM could be inferred. The solution showed no color due to reduced benzyl viologen, since this was immediately oxidized by oxygen. The measurements demonstrate that the activity is not inhibited by O_2. From EPR spectra (see below) the enzyme concentration in the extract was determined to be 10 μM. This means that the specific activity of the enzyme is about 7.7 U/mg. This is one to two orders of magnitude lower than the specific activity of standard [NiFe]-hydrogenases in hydrogen-consumption assays (100–800 U/mg; [1] and references therein).

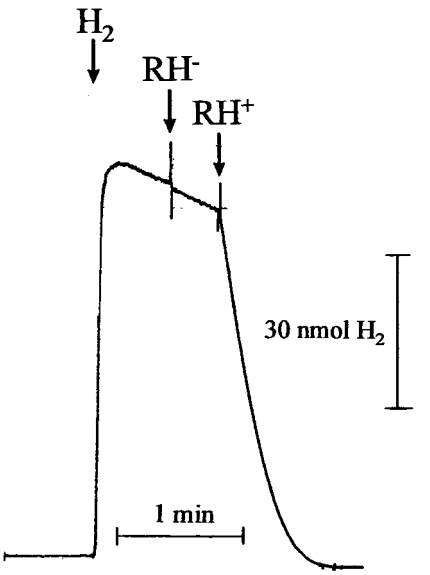

Fig. 1. Hydrogen uptake activity of extracts of RH^- and RH^+ cells (stored in liquid nitrogen), measured in air-saturated buffer (50 mM Tris-HCl, pH 8.0). The final benzyl viologen concentration was 25 mM.

3.2. EPR properties

Aerobic extract of RH^+ cells showed a faint signal due to nickel (Fig. 2, trace B; $g_{x,y}$ = 2.289, 2.168) resembling the Ni_r* signal of ready [NiFe]-hydrogenases. The spin concentration was about 0.8 μM. This signal was absent in extract of RH^- cells (Fig. 2, trace A). At g = 1.925 the $g_{x,y}$ lines of a reduced 2Fe cluster could be observed in both extracts. Its spin concentration in the RH^+ extract amounted to about 0.5 μM. The RH^+ extract was then treated with 100% H_2 and subsequently with 1% H_2, a procedure to optimize the nickel EPR signal of active hydrogenases [30]. This signal is here termed the Ni_a-C* signal, to emphasize the fact that this is a S = 1/2 signal (*) from Ni in active (a) enzyme, but that the formal valence state of nickel is still a matter of dispute. With extract from RH^+ cells this treatment caused the disappearance of the Ni_r* signal, whereas a strong signal (Fig. 2, trace C) typical

Table 1
Comparison of the ν(CN) and ν(CO) stretching frequencies of the sensor protein with those of the active [NiFe]-hydrogenases from *C. vinosum* and *D. gigas*

State	*A. eutrophus* RH	*C. vinosum*[a]	*D. gigas*[b]
Ni_a-S	2081/2073/1943[c]	2086/2074/1932	2085/2075/1934
Ni_a-C*	2084/2072/1962	2087/2074/1950	2086/2073/1952
Ni_a-SR[d]	not present	2073/2059/1936	2073/2060/1940

[a]From [39].
[b]From [40].
[c]The numbers in each row represent the stretching frequencies in cm^{-1} of the two CN^- groups (symmetric and antisymmetric vibrations), and of the CO molecule, respectively.
[d]State obtained under 100% H_2.

for Ni_a-C* appeared ($g_{x,y,z} = 2.191, 2.133, 2.010$). Its spin concentration was 10 μM.

Just as with all [NiFe]-hydrogenases, the RH in the Ni_a-C* state was light-sensitive at low temperatures. Illumination at 30 K resulted in the complete disappearance of the signal, and the simultaneous appearance of a signal with $g_{x,y,z} = 2.041$, 2.089, 2.244 (Fig. 2, trace D). The signal resembles the light-induced EPR signal in [NiFe]-hydrogenases (here termed Ni_a-L*), although the g values differ noticeably, especially the g_z value (2.244 versus 2.30). As with [NiFe]-hydrogenases, the light-induced changes reversed in the dark at 200 K (not shown).

When treated with 100% H_2 the sample showed an EPR spectrum (Fig. 2, trace E) identical to that obtained with 1% H_2 (Fig. 2, trace C). At 4.2 K and a high microwave power a splitting could be observed in the sample under 100% H_2 (not shown). Such a splitting is also detected in other hydrogenases [31–33] and is ascribed to interaction with the unpaired spin of the rapidly relaxing, reduced proximal [4Fe-4S] cluster. At a low microwave power the main signal showed no splitting.

3.3. FTIR spectra

Aerobic extract of RH^+ showed a clear FTIR spectrum (Fig. 3, trace B) with three absorption bands in the 2150–1900 cm^{-1} spectral region, just like [NiFe]-hydrogenases [6,34]. Extract of RH^- cells showed no such bands (Fig. 3, trace G). Treatment of RH^+ extract with 100% H_2 shifted the strong band from 1943 cm^{-1} to 1962 cm^{-1}, whereas the two weak bands were hardly displaced (Fig. 3, trace F). Incubation of the extract under several other reducing conditions (see legend to Fig. 3) did not evoke additional absorption bands; rather mixtures of the two states were observed (Fig. 3, traces C, D and E). Since the EPR spectrum of aerobic extracts showed signals of reduced 2Fe cluster (line at $g = 1.925$ in Fig. 2, traces A and B), the redox potential of this extract was apparently still rather low. We therefore added excess 2,6-dichlorophenol-indophenol ($E_o' = +230$ mV) to a sample such that its blue color could still be clearly seen in the FTIR cell before and after the measurement. The FTIR spectrum of this sample (Fig. 3, trace A) was exactly the same as that from untreated extract, showing that no further oxidation of the active site occurred. If H_2 was replaced by carbon monoxide a spectrum similar to Fig. 3 traces C and E was obtained, but no new bands appeared (not shown).

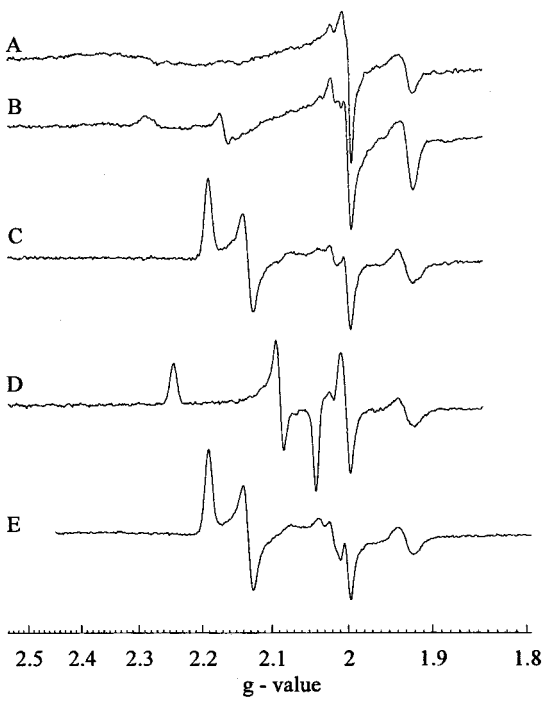

Fig. 2. EPR spectra of extracts of RH^- and RH^+ cells under various conditions. A: RH^- extract as prepared in air. B: RH^+ extract as prepared in air. C: RH^+ extract was incubated under 100% H_2 for 60 min at 35°C and subsequently flushed with 1% H_2, 99% He and frozen in liquid nitrogen in dim light after 60 min at 35°C. D: After illumination for 30 min at 30 K. E: The extract treated as in C and D was thawed, incubated under 100% H_2 for 30 min at 50°C and frozen in liquid nitrogen in the dark. EPR conditions: microwave frequency, 9416.9 MHz; microwave power incident to the cavity, 0.26 mW; modulation amplitude, 1.27 mT; temperature, 30 K. The same gain was used for all traces.

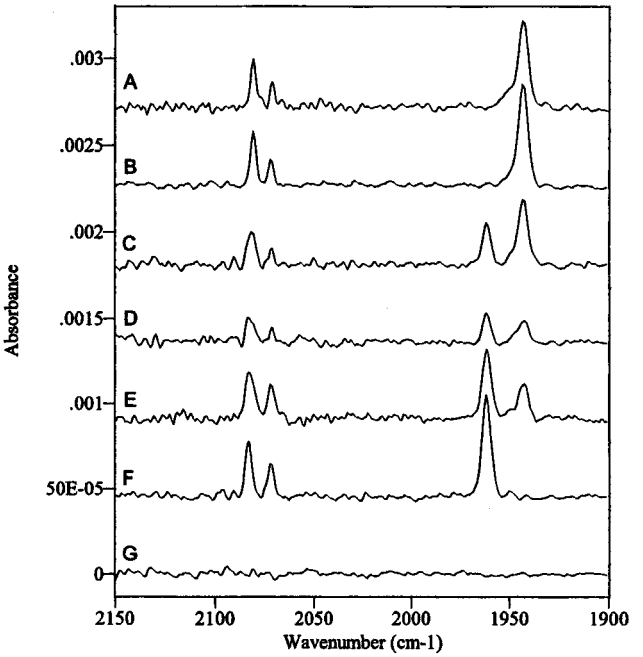

Fig. 3. Infrared spectra recorded at room temperature of extract of RH^+ cells under several conditions. A: Excess of 2,6-dichlorophenol-indophenol was added and after 60 min at room temperature the IR spectrum was recorded. The solution was still blue to the eye after this measurement. B: Aerobic extract without any treatment. C: Extract was evacuated and flushed with 100% H_2 at least 8 times. After 90 min at 35°C, H_2 was replaced by Ar and a spectrum was taken after 60 min at 35°C. D: Extract was mixed with 25 μM methyl viologen and 25 mM dithionite. A spectrum was taken after 30 min at room temperature. E: Extract was treated with H_2 as under C. Subsequently, H_2 was replaced by a mixture of 1% H_2/ 99% He and a spectrum was taken after 60 min at 35°C. F: Extract treated with 100% H_2 for 90 min at 35°C. G: Aerobic extract from RH^- cells. Spectra are averages of 1500 scans, except for B and G where 3000 scans were used.

4. Discussion

4.1. H_2-activating activity of the sensor

From Fig. 1 and other studies ([15]; Kleihues, Lenz, Bernhard and Friedrich, in preparation) it is clear that the RH can activate molecular hydrogen. There are three prominent differences with standard [NiFe]-hydrogenases: (i) contrast to most [NiFe]-hydrogenases, the RH in aerobic solution is al-

ways in an active state, as no lag phase was detected in any of the activity measurements; (ii) the RH is insensitive towards oxygen and is fully active in air-saturated assay buffer. With FTIR no binding of externally added CO was noticed; (iii) the RH contents as estimated from EPR and FTIR spectra, as compared to those from the *Chromatium vinosum* enzyme, are mutually consistent and corroborate the finding that the specific activity of the RH is much lower than that of standard [NiFe]-hydrogenases.

4.2. EPR spectra

In most [NiFe]-hydrogenases the active site can occur in five to seven different redox states [1]. The oxidized, aerobic sensor protein only showed a trace of a Ni_r* EPR signal (8% of the concentration of Ni_a-C*). This indicates that the majority of the protein molecules are present in an active, EPR-silent state comparable to the Ni_a-S state of hydrogenases. After reduction with H_2, the RH showed a typical Ni_a-C* signal, both under low (1%) and high (100%) H_2 partial pressures (Fig. 2, traces C and E). Apparently the Ni_a-C* state is not further reduced by increasing the H_2 partial pressure.

In the Ni_a-C* state, the active site in [NiFe]-hydrogenases contains a bound hydrogen species, which can be photodissociated at low temperature [35]. Illumination of Ni_a-C* at $T < 60$ K results in an EPR spectrum (Ni_a-L*) which is often a mixture of up to three different signals [35–37]. The main signal, produced in the *C. vinosum* enzyme at $T < 30$ K has $g_{x,y,z} = 2.295$, 2.135, 2.045, reflecting a considerable light-induced change at the nickel site. A similar change was observed after illumination of the RH (Fig. 2, trace D). The g_z value of the light-induced signal was 2.244, compared with 2.295 in the *C. vinosum* enzyme. This reflects distinct differences in the environment of the unpaired spin in the RH and in the *C. vinosum* hydrogenase. The light sensitivity indicates that, as in [NiFe]-hydrogenases, a photodissociable hydrogen species, e.g. a hydride, is bound to the active site. As the splitting of the Ni_a-C* signal (under 100% H_2) at 4.2 K was only well detected at high microwave powers, it is concluded that the proximal cluster was reduced in only a small fraction of the protein molecules. The signal of non-interacting Ni_a-C* is completely saturated at a high microwave power. The EPR measurements demonstrate that the RH occurs in only two states: oxidized (Ni_a-S) or reduced (Ni_a-C*).

4.3. FTIR spectra

The oxidized, aerobic sensor protein shows three bands, just like standard [NiFe]-hydrogenases [6,5,38–40]. In analogy, we ascribe the bands to the stretching frequencies of one CO (1943 cm^{-1}), and the symmetric and antisymmetric stretching frequencies of two nearly equivalent CN^- groups (2081 and 2073 cm^{-1}, respectively). Upon reduction with H_2, the ν(CO) stretch vibration shifts 19 cm^{-1} to higher frequencies, whereas the ν(CN) stretch vibrations are virtually unperturbed. None of the reducing or oxidizing conditions described in Fig. 3 resulted in the appearance of additional bands; only two states, sometimes as a mixture, could be detected. The states compare best with two of the three states observed in active [NiFe]-hydrogenases: the Ni_a-S (Fig. 3, traces A and B) and Ni_a-C* (Fig. 3, trace F) states. This is in complete agreement with the conclusion from the EPR data (Fig. 2). The IR frequencies of the RH and two well-studied [NiFe]-hydrogenases are compared in Table 1. The ν(CN) frequencies agree quite well; the ν(CO) stretch vibration of the RH is 11–12 cm^{-1} higher in frequency. It is noteworthy that in all three enzymes reduction of the Ni_a-S state to the Ni_a-C* state has virtually no effect on the ν(CN) bands, but increases the ν(CO) vibration frequencies to the same extent (approx. 18 cm^{-1}). This could point to a specific change in the coordination of Fe, opposite to the CO ligand.

5. Conclusions

We conclude that the RH contains an active site like that in standard [NiFe]-hydrogenases: a Ni-Fe center with two CN^- groups and one CO molecule bound to Fe. Contrary to these hydrogenases, the RH can occur only in two active states: Ni_a-S and Ni_a-C*. The latter state has a hydrogen species bound to the active site. The oxidized RH is always on alert to sense dihydrogen. With artificial electron donors or acceptors, the RH is able to reduce H^+ or to oxidize H_2, but not at high rates. The RH has only one binding site for H_2. Normal [NiFe]-hydrogenases are proposed to have two such sites [28], one of which (the one still vacant in the Ni_a-C* state) is believed to be involved in the real turnover activity of these enzymes. This site is apparently blocked in the RH, preventing reduction beyond the Ni_a-C* state. The altered protein environment presumably also prevents the reaction with oxygen or CO.

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