Biocatalysis

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Probing the Active Site of an O_2-Tolerant NAD^+-Reducing [NiFe]-Hydrogenase from *Ralstonia eutropha* H16 by In Situ EPR and FTIR Spectroscopy**

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[NiFe]-hydrogenases catalyze the reversible cleavage of dihydrogen into two protons and two electrons.[1] This process plays an important role in the energy metabolism of many microorganisms. For most [NiFe]-hydrogenases, the process of H_2 cycling is extremely sensitive to molecular oxygen as O_2 exhibits a high affinity to the active site. However, some organisms are capable of catalyzing H_2 cycling even at ambient oxygen levels.[2,3] Notably, the β-proteobacterium *Ralstonia eutropha* H16 (*Re*) harbors three different [NiFe]-hydrogenases, all of which display a remarkable oxygen-tolerance.[2–4] The underlying molecular mechanisms are not yet fully understood. For the regulatory hydrogenase (RH) of *Re*, a narrow gas tunnel is thought to restrict O_2 access to the active site.[4] The *Re* membrane-bound hydrogenase (MBH) has a high redox potential FeS cluster in close proximity to the active site,[5] a property that might be related to the observation that O_2-inhibited MBH re-activates rapidly at high potentials.[6] The soluble hydrogenase (SH) of *Re* is a cytoplasmic NAD^+-reducing six-subunit enzyme that is closely related to cyanobacterial bidirectional [NiFe]-hydrogenases.[2,7] For purified SH, a modified catalytic site was proposed on the basis of numerous biochemical and spectroscopic studies.[2,8,9] In contrast to "standard" [NiFe]-hydrogenases, in which the active site iron is kept in the low-spin iron(II) state by one carbonyl and two cyanide ligands, Fourier transform infrared (FTIR) spectroscopy and concomitant chemical analysis suggested one additional cyanide bound to each metal ion of the catalytic center. The nickel-bound cyanide ligand has been proposed to prevent the formation of the so-called Ni_u-A state, which is the most oxidized, O_2-inactivated state in [NiFe]-hydrogenases.[9]

Controversial results have been obtained concerning the occurrence of paramagnetic nickel states in the SH. The Ni_r-B state, representing an oxidized active site carrying a hydroxide ligand in the bridging position between Ni and Fe, was not observed for the purified protein. However, studies on SH preparations treated with an excess of NADH or dithionite revealed electron paramagnetic resonance (EPR) signals and FTIR bands attributable to the catalytic intermediate Ni_a-C and the light-induced, non-physiological, Ni_a-L state.[10,11] However, these redox states, which are common for anaerobic "standard" [NiFe]-hydrogenases, were later proposed to be not involved in the SH catalytic cycle.[2,12] Instead, a reaction mechanism was suggested in which the Ni remains in the EPR-silent Ni^{II} state throughout the catalytic cycle while redox changes at the active site are solely reflected by wavenumber shifts of the CN stretching vibration originating from the nickel-bound cyanide. The fully reduced, EPR-silent Ni_a^{II}-SR states, which comprise up to three subpopulations and which are normally detected in catalytically active "standard" [NiFe]-hydrogenases, could not be detected for the SH upon reduction with H_2 or/and NADH.

Albeit O_2-tolerant in catalysis, the SH can be inactivated by oxygen, as purified SH requires reductive activation by catalytic amounts of either NADH or NADPH.[2,12] Information on additional SH cofactors was obtained by EPR spectroscopic experiments, revealing signals for a reduced [2Fe2S]-cluster and a flavin radical (FMN semiquinone) generated after incubation with H_2 in the presence of catalytic amounts of NAD(P)H or by the addition of excess NADH. Only rigorous reduction by dithionite revealed additional EPR signals attributable to one [4Fe4S] cluster.[2]

To date, spectroscopic studies on the *Re* SH have been performed exclusively on purified enzyme samples.[2,9] In the present study, we have investigated the SH for the first time in situ, that is, as a constituent of the cytoplasm in whole cells, by using a combined EPR and FTIR spectroscopic approach. All experiments were performed with a wild-type derivative of *Re* H16 that solely synthesizes the soluble hydrogenase. The genes encoding the active site-containing large subunits of the two other *Re* hydrogenases were inactivated by markerless inframe deletion. Thus, SH biosynthesis should not be affected in this strain, and any interference of the SH-related

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spectroscopic signals with those from the RH and the MBH can be excluded.

Figure 1 shows the EPR spectra of differently treated *Re* cells. Trace A is the spectrum of the freshly harvested cells at $T = 35$ K. In the low-field region, strong Ni signals are visible

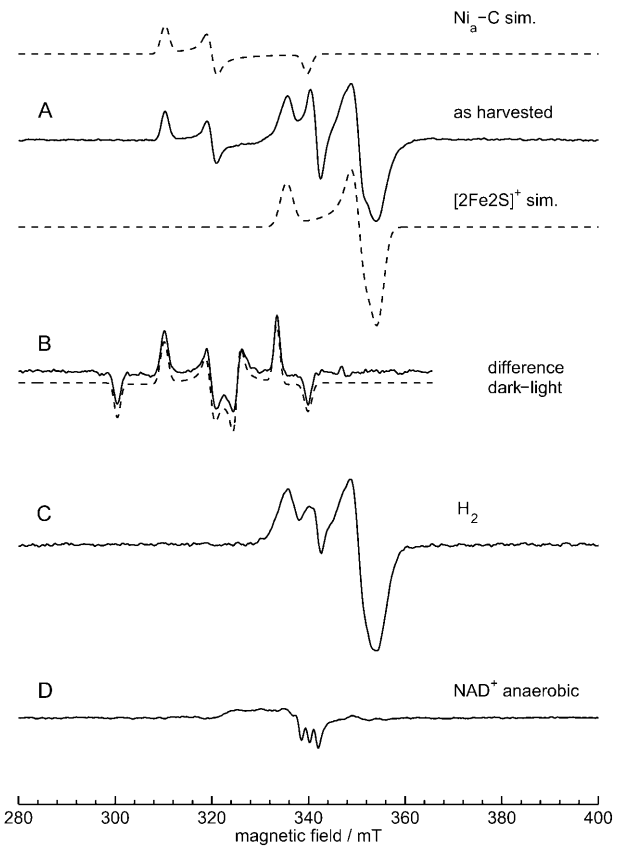

Figure 1. In situ EPR spectra of the *Re* SH recorded at $T = 35$ K (solid lines) and the corresponding simulations (dashed lines). A) cells as-harvested, B) difference between dark-adapted minus light exposed cells, C) cells as-harvested, exposed to H_2, D) CTAB-treated cells oxidized anaerobically with NAD$^+$.

with well-resolved g_x and g_y components. The g-values deduced from simulations are 2.20, 2.14, and 2.01 with a linewidth of 1.8 mT and can be attributed to the Ni$_a$-C redox state, formally Ni^{III}, as found in "standard" [NiFe]-hydrogenases.[13] Additionally, signals for FMN ($g = 2.00$) and a [2Fe2S]$^+$ cluster were detected. These observations are consistent with the reducing conditions in the cytoplasm. A relative spin quantification was performed by comparing the double-integrated simulations of Ni$_a$-C and the quantitatively reduced [2Fe2S]$^+$ cluster, revealing approximately 60 % Ni$_a$-C in freshly harvested cells. In accordance with observations for "standard" [NiFe]-hydrogenases, the Ni$_a$-C state in the SH was converted completely into Ni$_a$-L by white-light illumination at $T = 80$ K for 30 min.[14] 10 min of dark adaptation at $T > 100$ K led to a complete back conversion into Ni$_a$-C. Simulation of the difference spectra at 35 K revealed g values of 2.27, 2.10, and 2.05 with a linewidth of 1.5 mT for the Ni$_a$-L state (Figure 1 B).

Upon incubation with H_2 for 30 min, the Ni$_a$-C signals disappeared (Figure 1 C) whereas the [2Fe2S]$^+$-cluster and FMN signals persisted. This finding indicates that the active site has been further reduced in a one-electron process to the Ni$_a$-SR state(s), which are commonly observed in "standard" [NiFe]-hydrogenases. On the other hand, oxidation of SH-containing cells, which were permeabilized by treatment with cetyl trimethylammonium bromide (CTAB) and subsequently incubated with an excess of NAD$^+$ under anaerobic conditions, led to the disappearance of any Ni- and FeS-related EPR signals (Figure 1 D). Only background signals from unknown cellular paramagnetic centers remained visible. Signals attributable to the oxidized Ni$_r$-B or Ni$_u$-A species, in which Ni is present in the paramagnetic Ni^{III} form, could not be identified in our experiments. Similar results were obtained upon oxidation with air (not shown).

The corresponding FTIR data are displayed in Figure 2. As a result of whole cells being used for the measurements, the CO and CN band intensities in the FTIR spectra are relatively low and superimposed by a strongly contoured baseline. Thus, for a more reliable identification of the various bands we have used the second-derivatives of the spectra in which the individual bands appear as negative peaks. Figure 2 A depicts the FTIR spectrum of freshly harvested *Re* cells. The spectrum is dominated by a CO absorption at

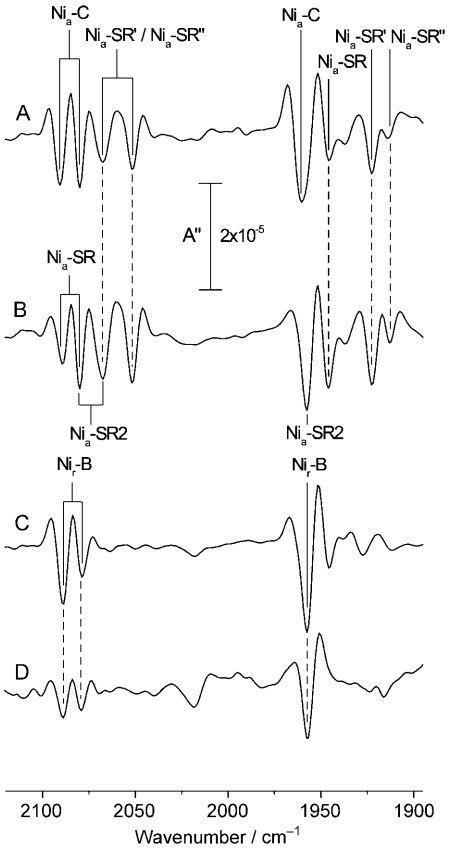

Figure 2. In situ FTIR spectra (2nd derivative) of the soluble hydrogenase: A) cells as harvested, B) after 30 min incubation under 1 bar H_2, C) oxidized with NAD$^+$ under anaerobic conditions, and D) oxidized under aerobic conditions.

1961 cm^{-1} and two corresponding CN stretching bands at 2080 and 2091 cm^{-1}. These bands are assigned to the Ni_a-C state of the SH, which appears to represent the main fraction, consistent with the EPR spin quantification. The Ni_a-C-related stretching vibrations are slightly shifted to lower wavenumbers (2–5 cm^{-1}) compared to previous investigations with purified enzyme at cryogenic temperatures.[9] These deviations might be to due to temperature-dependent changes in the hydrogen-bonding network and/or different pH values in the cytoplasm and the buffer of the purified enzyme.[15,16] Furthermore, absorption bands in the lower frequency range of the CO and CN stretching vibrations were observed at 1913, 1922, 2052, and 2068 cm^{-1}. The 2052 and 2068 cm^{-1} bands are broadened by an overlap of adjacent absorptions. Upon incubation with 1 bar H_2, these bands increased significantly in intensity (Figure 2B) and, therefore, are assigned to the reduced species Ni_a-SR′ and Ni_a-SR″, respectively. Bands at 1946, 2080, and 2090 cm^{-1} were attributed to the Ni_a-SR state. Regarding the CO stretching vibrations, these assignments confirm previous spectro-electro-chemical FTIR data of purified *Re* SH recorded under reductive conditions at −391 mV versus the normalized hydrogen electrode (NHE).[12] Furthermore, the bands at 1958, 2068, and 2080 cm^{-1} are tentatively attributed to a further reduced, EPR-silent Ni_a-SR2 species. The assignment of the individual reduced species is based on recent FTIR spectroscopic studies of the bidirectional hydrogenase of *Synechocystis* sp. PCC 6803 and *Re* MBH[6,7] (Table 1). Incubation of CTAB-treated cells with either an excess of NAD^+ under anaerobic conditions (Figure 2C) or with air (Figure 2D) revealed three bands at 1957, 2079, and 2089 cm^{-1}. In accordance with data obtained for the bidirectional hydrogenase from *Synechocystis* sp. these bands are attributed to a "Ni_r-B-like" state, which is, however, EPR-silent. Such a "Ni_r-B-like" species, as well as the distinct reduced Ni_a-SR2 state, is exclusively found in [NiFe]-hydrogenases equipped with a heterodimeric NADH:acceptor oxidoreductase module.[7] An overview of the various active site states is given in the Supporting Information, Scheme S1.

Table 1: CO and CN stretching-mode frequencies [cm^{-1}] of all redox states observed in SH-containing cells and for purified SH.[12][a]

Redox state	ν(CO)	ν(CN)	
Ni_r-B-like	**1957** (1957*)	**2079** (2076*)	**2089** (2088*)
Ni_a-C	**1961** (1968*)	**2080** (2079*)	**2091** (2093*)
	1963 at 35 K[12]	2084 at 35 K[12]	2096 at 35 K[12]
Ni_a-SR	**1946** (1948°)	**2080** (2068°)	**2090** (2087°)
	1945 (−391 mV)[12]	n.d.	n.d.
Ni_a-SR′	**1922** (1926°)	**2052** (2049°)	**2068** (2075°)
	1921 (−391 mV)[12]	n.d.	n.d.
Ni_a-SR″	**1913** (1919°)	**2052** (2046°)	**2068** (2071°)
	1912 (−391 mV)[12]	n.d.	n.d.
Ni_a-SR2	**1958** (1955*)	**2068** (2063*)	**2080** (2079*)

[a] Numbers in parentheses correspond to the bidirectional hydrogenase from *Synechocystis* sp. PCC 6803 (*) and the MBH from *R. eutropha* H16 [°].[7,17,18] Abbreviations from [15]: r = ready, a = activated, B and C refer to an oxidized and intermediate EPR-active state, respectively; R, R2, reduced states; S, EPR-silent; n.d. not determined in [12].

The EPR spectroscopic data presented in our study showed that a major fraction of the *Re* SH in freshly harvested cells resides in the Ni_a-C state. Accompanying FTIR spectroscopic investigations of whole cells led to the identification of the EPR-silent catalytically active Ni_a-SR and Ni_a-SR2 states, which were previously detected in other [NiFe]-hydrogenases.[5,7,15–18] The Ni_a-C⇌Ni_a-L and Ni_a-C⇌Ni_a-SRx transitions turned out to be reversible, indicating a fully intact active site (see Supporting Information). Anaerobic as well as aerobic oxidation led to the complete disappearance of all Ni_a-C related bands. Instead, a "Ni_r-B-like" state was obtained, which is identified by one CO and two CN stretching modes at specific band positions. However, it is unclear whether these spectral features represent a real Ni_r-B state, which is EPR-silent, owing to spin-couplings with other paramagnetic centers, or just a "Ni_r-B-like" species with a formal Ni^{II} state. Two further cell treatments, including the permeabilization of aerated cells by three consecutive freeze–thaw cycles without using any detergents, also resulted in the formation of this particular state (see Supporting Information). Notably, it was possible to recover the catalytically active states by incubating the oxidized enzyme with H_2. A fully reversible redox-behavior of the SH, as a consequence of exchanging the gas-atmosphere from inert to oxidizing conditions and vice versa, was also shown for *Re* grown under lithoautotrophic conditions (see Supporting Information).

The current in situ study indicates that the *Re* SH active site contains a "standard set" of non-proteic, inorganic ligands, that is, one CO and two CN^-. This observation is in sharp contrast to previous results obtained for purified SH isolated from the wild-type strain *Re* H16, for which two additional cyanide ligands were proposed to be constituents of the active site.[2,8,9,12] Consequently, our results implicate that the mechanism of oxygen tolerance, which was developed on the basis of purified SH, should be reconsidered. The present case demonstrates that, as expected, the cytoplasmic constitution has a major influence on the redox properties of the SH. This influence includes the interaction of the enzyme with reductants (e.g. H_2, NAD(P)H), oxidants (e.g. $NAD(P)^+$, O_2), protons (that is, changes in pH value), salts, osmolytes, and other proteins. In a more general sense, the present results demonstrate that the functional and structural integrity of enzymes might require the preservation of the native environment, thereby representing new challenges for in situ spectroscopy.

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