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Biocatalysis

Monitoring Catalysis of the Membrane-Bound Hydrogenase from *Ralstonia eutropha* H16 by Surface-Enhanced IR Absorption Spectroscopy **

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[NiFe] hydrogenases constitute a class of enzymes that catalyze the heterolytic splitting of molecular hydrogen (H_2) as well as the reverse reaction, the reduction of protons to H_2.[1] The catalytic site is a bimetallic Ni/Fe complex bridged by four conserved cysteine residues. Moreover, one CO and two CN^- ligand bind to iron as rather unusual exogenous ligands. Whereas most of these enzymes are catalytically active only under strictly anaerobic conditions, the [NiFe] hydrogenases of *Ralstonia* species have a remarkable oxygen tolerance, the origin of which is not yet fully understood on the molecular level.[2–4] This particular oxygen tolerance has prompted considerable research effects in the past that were motivated by the interest in elucidating the catalytic mechanism of these enzymes and by their potential importance for biotechnological energy storage and conversion. Such applications require the immobilization of the enzymes on electrically conducting supports under preservation of the native structure and function.[5] In fact, the first demonstrations of successful immobilization have been reported for the membrane-bound hydrogenases of *Ralstonia* species. These enzymes were attached to pyrolytic graphite electrodes to build simple enzymatic fuel cells that operate even with H_2 concentrations lower than 3% in air.[2,3]

To investigate the performance of enzymes immobilized on surfaces, it is highly desirable to develop an experimental approach that is capable of probing the molecular structure of the active site and their changes during the catalytic processes in situ. IR spectroscopy is one of the main techniques used for the identification of the various states of the catalytic cycle by probing the stretching modes of the CO and CN^- ligands of the Ni–Fe active site. The frequencies of these modes sensitively reflect changes of the electron density within the catalytic center caused by alterations of the metal oxidation state, ligation pattern, and cofactor–protein interactions. Therefore, IR spectroscopy has been widely used for characterizing the enzymatic process of hydrogenases in bulk solution and, in conjunction with EPR spectroscopy, it has provided important insights into the mechanism of the enzymatic process.[6–8]

The main drawback of conventional IR spectroscopy is the relatively low sensitivity, which is not sufficient for studying immobilized enzymes. Surface-enhanced infrared absorption (SEIRA) spectroscopy promises to overcome this limitation, as the IR absorption can be enhanced by up to two orders of magnitude for proteins immobilized on gold surfaces.[9,10] SEIRA spectroscopy has already been successfully applied to monitor protein immobilization on gold electrodes and redox-linked structural changes of proteins under stationary conditions, and more recently, in the time-resolved domain.[11–14] Herein, this technique is applied for the first time to a hydrogenase, namely the membrane-bound hydrogenase of *Ralstonia eutropha* H16 (*R.e.* MBH), attached to a gold surface. Unlike previous studies of cofactor–protein complexes, the SEIRA spectroscopic analysis of hydrogenases is not restricted to changes of the protein structure, but allows the direct observation of the characteristic marker bands of the catalytic site in a spectral window, without interference from IR absorption bands of the protein.

SEIRA experiments were carried out in a Kretschmann ATR configuration using a semicylindrical silicon crystal coated with a gold film by electroless deposition.[15] The gold surface was subsequently covered by a self-assembled monolayer of nickel nitrilotriacetic acid (Ni-NTA)[11,16] for affinity binding of the *R.e.* MBH, which was modified by a His-tag at the C-terminus of the electron-transferring subunit of the dimeric enzyme (for further experimental details, see Supporting Information). This strategy ensured a uniform and unidirectional orientation of the immobilized protein. The efficient immobilization is documented by the SEIRA spectrum obtained under an argon atmosphere (Figure 1). The presence of the strong amide I and II bands indicated a high degree of coverage. A closer inspection of the spectral region between 1850–2150 cm^{-1} reveals the weak but clearly detectable bands of the stretching modes of the diatomic ligands coordinated to the active site, iron. A dominant CO stretching mode is present at 1948 cm^{-1}, with the conjugate CN^- stretching vibrations at 2098 and 2081 cm^{-1}. This band pattern is characteristic of the so-called "ready", Ni_r-B state (Figure 2, Table 1).[7,17] Upon immobilization, both the amide and the CO stretching bands increased concomitantly with

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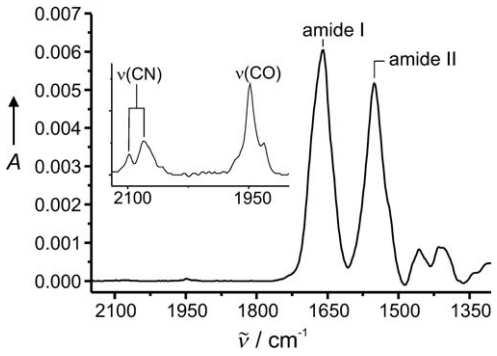

Figure 1. SEIRA spectrum of the *R.e.* MBH bound by a His-tag to the Ni-NTA-modified gold surface. Inset: expanded spectral region between 1850–2150 cm^{-1}, which includes the stretching modes of the CO and CN$^-$ ligands.

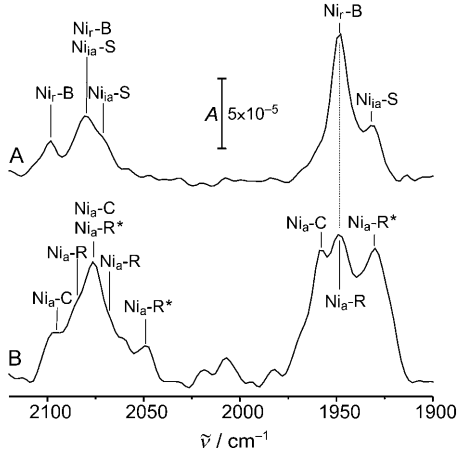

Figure 2. SEIRA spectrum of the *R.e.* MBH bound by a His tag to the Ni-NTA-modified gold surface A) before and B) after exposure to H_2 (buffered at pH 5.5). "Ni$_a$-R*" refers to the substates Ni$_a$-R′ and Ni$_a$-R″ that give rise to closely spaced CO and CN stretching modes.

Table 1: CO and CN$^-$ stretching-mode frequencies (in cm^{-1}) of various states of *R.e.* MBH.[17] [a]

Redox state	$\tilde{\nu}$(CO)	$\tilde{\nu}$(CN$^-$)	
Ni$_r$-B	1948	2081	2098
Ni$_{ia}$-S	1930	2060	2076
Ni$_r$-S	1936	2075	2093
Ni$_a$-C	1957	2075	2097
Ni$_a$-RS	1948	2068	2087
Ni$_a$-RS′	1926	2049	2075
Ni$_a$-RS″	1919	2046	2071

[a] Band positions were determined for *R.e.* MBH in solution at pH 5.5. The oxidized states (Ni$_r$-B, Ni$_r$-S, Ni$_{ia}$-S) refer to the as-isolated form of the enzyme under aerobic conditions, whereas the reduced states are obtained with H_2 at a pressure of 1 bar. Ni$_a$-C refers to reduced intermediate state, and Ni$_a$-RS, Ni$_a$-RS′, Ni$_a$-RS″ comprise a mixture of the fully reduced states. ia = inactive, r = ready, a = activated, B,C = standard nomenclature[7,8] for an oxidized and intermediate EPR-active redox state, respectively, R = fully reduced states, S = EPR silent.

time, indicating a uniform orientation and preservation of the catalytic site structure of the adsorbed enzyme (see Supporting Information).

The weak shoulder at 1930 cm^{-1} originates from the CO stretching mode of the inactive Ni$_{ia}$-S state, for which the corresponding CN stretching modes are known to appear at 2076 and 2060 cm^{-1} and thus partly overlap with the CN stretching modes of Ni$_r$-B. On the basis of the integral intensities of the CO stretching bands, the relative amounts of Ni$_r$-B and Ni$_{ia}$-S is estimated to be 70% and 30%, respectively. Injection of H_2 into the SEIRA cell leads to a decrease of the bands originating from the Ni$_r$-B state and an increase of bands characteristic of the active reduced states that are involved in the catalytic cycle.[18–20] At 1 bar H_2 and a pH value of 5.5, the redox potential of H_2 was calculated to be −320 mV versus the normal hydrogen electrode (NHE), which is sufficiently negative to convert most of the oxidized enzyme into the reduced states. These states were identified based on the comparison with the IR transmission spectrum of the H_2-reduced *R.e.* MBH bound to the inner membrane, for which a detailed band assignment has been provided in a recent IR and EPR study.[17] Moreover, the comparison of the IR and SEIRA spectra also displayed far-reaching similarities and good agreement with the "oxidized" minus "H_2-reduced" IR and SEIRA difference spectra.

These results demonstrate that H_2 treatment of the oxidized enzyme attached to the coated gold surface induces the same transformation as previously observed for the membrane-attached protein in the bulk. Furthermore, the distribution of the various reduced species is quite similar (Figure 3A, B). Among them is the EPR detectable inter-

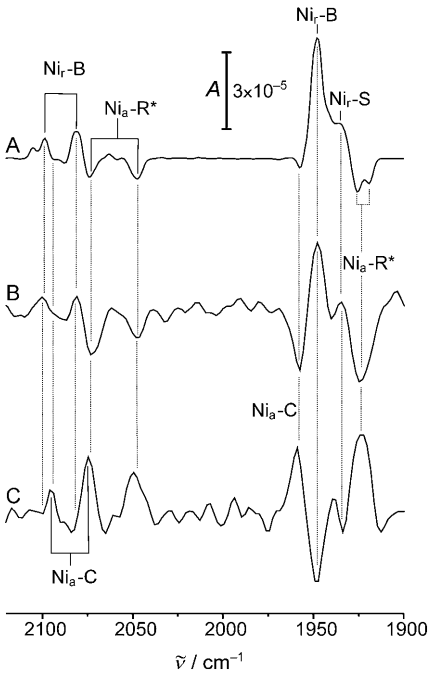

Figure 3. IR and SEIRA difference spectra of the *R.e.* MBH. A) IR transmission difference spectrum of MBH attached to the cytoplasmic membrane in the bulk phase (oxidized minus H_2 reduced). B,C) SEIRA difference spectrum of the *R.e.* MBH bound by a His-tag to the Ni-NTA gold surface: oxidized minus H_2-reduced (B), H_2-reduced minus argon re-oxidized (C). "Ni$_a$-R*" refers to the substates Ni$_a$-R′ and Ni$_a$-R″ that give rise to closely spaced CO and CN stretching modes. The intensity of spectrum A is divided by a factor of 20.

mediate Ni_a-C that can be identified on the basis of the IR bands at 1957 (CO), 2075 (CN−), and 2097 (CN−) cm^{-1}. The frequency of the CO stretching mode of the EPR-silent Ni_a-R state coincides with that of Ni_r-B at 1948 cm^{-1}, such that IR intensity at this frequency remains at the same level, even after complete reduction of the Ni_r-B state (Figure 2). The CN stretching modes of Ni_a-R are expected to be at 2068 and 2087 cm^{-1} and in fact can be observed as shoulders of the 2081 cm^{-1} CN stretching of Ni_r-B. The closely spaced CO bands of the two Ni_a-R′ and Ni_a-R″ states are only resolved in the IR difference spectra that were obtained with 2 cm^{-1} resolution compared to the resolution of 4 cm^{-1} of the SEIRA spectra (Figure 3 A, B). The corresponding CN stretching modes are at 2049 and 2075 cm^{-1} for Ni_a-R′ and at 2046 and 2071 cm^{-1} for Ni_a-R″, and partly overlap with the CN stretching of Ni_a-C at 2075 cm^{-1}.

The spectral changes of the enzyme immobilized on the gold surface that are induced by H_2 reduction are reversible, as demonstrated by subsequent incubation with argon. The SEIRA difference spectrum "H_2-reduced" minus "argon, re-oxidized" (Figure 3 C) are nearly a mirror image of the "oxidized" minus "H_2-reduced" difference spectrum (Figure 3 B). The difference spectra also clearly reveal the presence of another oxidized species, the so called "ready" silent Ni_r-S state, with a CO stretching vibration at 1936 cm^{-1}.

The SEIRA spectroscopic results in this study demonstrate that His-tag-mediated immobilization of engineered hydrogenases allows the binding of the enzymes to gold surfaces without affecting the native protein structure and the reactivity towards hydrogen. Further studies, using the metal support as an electrode, will be directed to the optimization of the electronic coupling of the surface with the catalytic center of the immobilized enzyme. This is a prerequisite for optimizing the functioning of hydrogenase-based bioelectronic devices. In this respect, stationary and time-resolved SEIRA spectroscopy represent an indispensable tool for in situ monitoring of structural changes within enzymes during catalysis and provide information that is complementary to that obtained from thin-protein-film voltammetry and related methods.[4,21]

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