Biocatalysis

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SEIRA Spectroscopy of the Electrochemical Activation of an Immobilized [NiFe] Hydrogenase under Turnover and Non-Turnover Conditions**

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[NiFe] hydrogenases are a family of metalloenzymes that catalyze the reversible splitting of molecular hydrogen into protons and electrons. These enzymes contain an electron-transfer chain of FeS clusters linking the external redox partner with the active site, a [NiFe] center. This bimetallic complex includes two terminal cysteine residues bound to the Ni atom, three diatomic inorganic ligands (one CO and two CN^-) bound to the Fe atom, and two cysteine residues bridging the Ni and Fe atoms.[1,2] The [NiFe] center may exist in various states, differing with respect to the oxidation state of the Ni atom and the nature of the exogenous ligand bridging the Ni and Fe atoms. Most of the information about the active-site structure in these states has been obtained by EPR and IR spectroscopy, leading to a better understanding of the catalytic processes in the hydrogen metabolism of (an)aerobic microorganisms.

In recent years, the interest in these enzymatic processes has dramatically increased in view of possible applications of hydrogenases as catalysts for biotechnological applications using hydrogen as an energy source.[3] To exploit the full potential of such enzymes in this field, they have to be immobilized on electrodes under conditions that preserve the native structure and function, and ensure an efficient electrical communication between the catalytic center and the conducting support materials. In recent years, promising results have been obtained demonstrating catalytic activity of immobilized hydrogenases on graphite electrodes and thus the proof-of-principle for a hydrogenase/laccase biofuel cell.[4]

Since EPR and conventional IR spectroscopy are not applicable to the enzymes immobilized on electrodes, the performance of such devices is usually monitored by electrochemical methods, specifically protein-film voltammetry (PFV). These techniques probe the electrical communication between the electrode and the enzyme but do not provide insight into mechanistic details of the catalytic processes and the nature of the species involved. Such information, however, is essential for improving hydrogenase-based bioelectronic devices. In this respect, surface-enhanced infrared absorption (SEIRA) spectroscopy may provide a promising alternative because it selectively enhances the IR bands of proteins immobilized on biocompatibly coated Au electrodes. Specifically SEIRA spectroscopy allows the detection of the CO and CN stretching vibrations of the active site in hydrogenases in different redox states, a prerequisite for monitoring in situ their catalytic processes.[5,6]

A key step for the biocatalytic process is the activation of "standard" (i.e. oxygen-sensitive) [NiFe] hydrogenases. These commonly exist in the as-isolated form as a mixture of two oxidized inactive states, in which presumably a hydroperoxide (Ni_u-A) and a hydroxide group (Ni_r-B) serve as the bridging ligand between the Ni and the Fe.[7,8] Reduction of Ni_u-A and Ni_r-B leads to the same active state (Ni_a-S), albeit via different pathways. Herein, we have employed SEIRA spectroscopy to study the activation of such an anaerobic "standard" [NiFe] hydrogenase from *Desulfovibrio vulgaris* Miyazaki F (*Dv*MF) on an Au electrode. In contrast to our previous studies, in which only H_2-induced activation of the immobilized enzyme could be monitored,[5,6] we report, for the first time, a SEIRA spectroscopic analysis of the electrochemical activation of the enzyme under turnover and non-turnover conditions (i.e., in the presence and absence of substrate, respectively).

SEIRA spectra were measured in the attenuated total reflection (ATR) mode using a silicon prism coated by a nano-structured Au layer. The Au-surface was functionalized with a self-assembled monolayer (SAM) of 6-amino-1-hexanethiol to establish electrostatic binding of the enzyme through its small subunit that accommodates the FeS clusters. The Au serves both as an amplifier for the spectroscopic signal and as a working electrode. An essential improvement of the experimental setup was achieved by placing the spectrometer inside an anaerobic tent (see SI 1 in the Supporting Information), because even trace amounts of oxygen can cause the irreversible degradation of the active site upon applying an electrode potential.[6]

The various redox states of hydrogenases can be identified on the basis of the characteristic IR signatures of the corresponding stretching modes of the CO and CN^- ligands coordinated to the active site. Of these bands, the CO stretching is the most prominent. SEIRA spectra in the CO stretching region obtained during the redox titration of the immobilized enzyme under an argon atmosphere are shown

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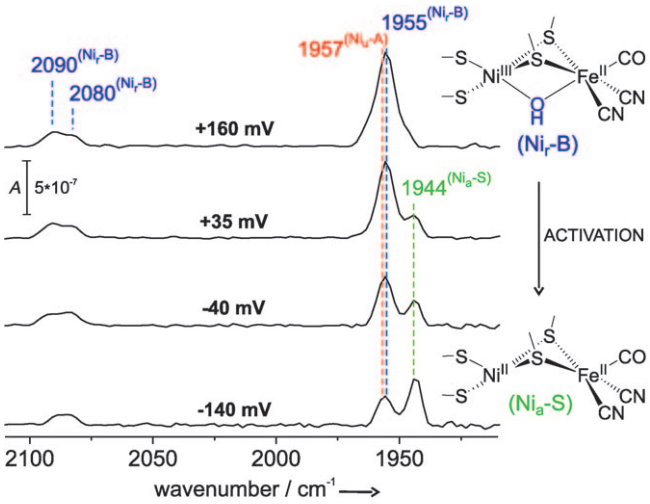

Figure 1. Potential-dependant SEIRA spectra of the [NiFe] hydrogenase from *Dv*MF immobilized on an Au electrode, which was chemically modified with a SAM of 6-amino-1-hexanethiol. Experiments were performed in 50 mM acetate buffer, pH 5.5, at 25 °C under argon atmosphere. Potentials are referenced versus the standard hydrogen electrode (SHE). The electronic and structural changes at the [NiFe] active site induced by an electrochemical activation are shown on the right.

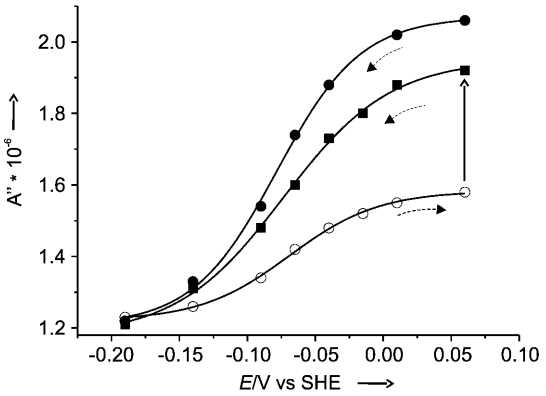

Figure 2. Nernstian fits (solid lines) of the second derivative of the absolute SEIRA spectra shown in Figure 1. (●) redox titration from positive to negative potentials, (○) redox measurements in the reverse direction. Although the original signal could not be fully recovered, $E_{1/2}$ and n values obtained from the fit are the same as for the direct (●) and reverse signal (○). The vertical arrow indicates a recovery of the original signal after holding the potential at +160 mV for 18 h. (■) subsequent, second redox titration (from positive to negative potentials).

Table 1: Comparison between $E_{1/2}$ and E_{switch}.[a]

$E_{1/2}$	n	E^{SEIRA}_{switch}	E^{CA}_{switch}	Δ_1[b]	Δ_2[c]
−64(±12)	0.8(±0.2)	−16(±14)	−7(±15)	48(±2)	9(±1)

[a] $E_{1/2}$ and E^{SEIRA}_{switch} were obtained by fitting the second-derivative peak intensities of the absolute SEIRA spectra. E^{CA}_{switch} was estimated by fitting the I versus E curves obtained at different potentials in a series of CA experiments performed simultaneously with the SEIRA measurements. In this case the potential was set at the desired value for 10 min, and when a stable current was detected (used to obtain the I versus E plot, which is needed to determine E^{CA}_{switch}), the SEIRA spectrum was acquired. Potentials are reported in mV and referenced versus the SHE. The corresponding error is given in parenthesis. [b] $\Delta_1 = E^{SEIRA}_{switch} - E_{1/2}$. [c] $\Delta_2 = E^{CA}_{switch} - E^{SEIRA}_{switch}$.

in Figure 1. A reliable analysis of the CN stretching region (2050–2110 cm^{-1}) is not feasible owing to the significantly weaker band intensities and band overlaps. At +160 mV (versus the standard hydrogen electrode (SHE)), the SEIRA spectrum displays a CO stretching mode at 1956 cm^{-1}, that comprises a superposition of the Ni_u-A and the Ni_r-B states. Stepwise lowering of the applied potential, leads to an intensity decrease of the 1956 cm^{-1} band and a concomitant increase of a band at 1944 cm^{-1}, originating from the Ni_a-S state. At −140 mV the band intensity at 1944 cm^{-1} reaches a plateau and does not increase further when the potential is set to −190 mV (data not shown). The observed potential-dependent spectral changes can be attributed to the electrochemical conversion of Ni_r-B into Ni_a-S, which involves the reduction of Ni^{3+} to Ni^{2+}, the protonation of the bridging OH^-, and the subsequent release of the formed H_2O molecule (Figure 1).[9] Furthermore, the band intensity at 1957 cm^{-1} that persists even at −190 mV is attributed to residual Ni_u-A state that cannot be fully converted into Ni_a-S at this potential.[6] Increasing the potential back to +160 mV leads to a reversal of the intensity changes, although the original intensity could not be completely recovered even after 120 min. However, an almost complete re-oxidation of Ni_a-S to Ni_r-B is achieved upon setting the electrode potential at +160 mV for 18 h, suggesting a kinetic hindrance of the underlying processes.

A quantitative analysis of the redox transition can be carried out on the basis of the potential-dependence of the intensity changes at 1956 cm^{-1} or, as shown in Figure 2, on the second derivatives of the absolute spectra. Fitting the Nernst equation to the resultant sigmoid-shaped curve reveals a nearly ideal behavior for an one-electron redox couple (n = 0.8) and a midpoint potential ($E_{1/2}$) of −64 mV (Table 1), which is very close to the value determined in solution for the Ni_r-B/Ni_a-S redox transition under similar experimental conditions (−58 mV at pH 5.5).[6] This result and the agreement of the band positions in the SEIRA and IR spectra imply that the structure of the active site and its adjacent protein environment are not perturbed in the immobilized enzyme. Furthermore, these findings demonstrate a good electrical coupling of the enzyme with the electrode, allowing for a reversible cycling between the conjugate redox states. Thus, for the first time, it was possible to assign an experimentally determined midpoint potential of an immobilized hydrogenase to a specific redox couple (Ni_rB/Ni_aS) based on their characteristic vibrational signatures. It should be noted that the midpoint potentials of immobilized enzymes under non-turnover conditions can also be obtained by PFV,[10,11] for [NiFe] hydrogenases (*Allochromatium vinosum* and *Desulfomicrobium baculatum*), however, to date only a redox transition of the FeS clusters—but not of the active site—has been identified.[12,13] For all other [NiFe] hydrogenases, including the *Dv*MF, a non-turnover signal could not be detected even in presence of inhibitors, such as CO.[14]

Now we consider the redox transitions under turnover conditions measured in the presence of H_2. A characteristic electrochemical quantity that is usually obtained by PFV, for

hydrogenases during their catalytic cycling is the so-called switch potential (E_{switch}). It is defined as the potential of maximum slope in the reductive activation direction, and is determined from a derivative plot of the cyclic voltammetric (CV) trace (see SI 2 in the Supporting Information).[15] The interpretation of this parameter is still under debate; in fact, although E_{switch} and $E_{1/2}$ are thought to refer to the same redox transition,[15,16] the underlying processes are different. Under argon atmosphere (non-turnover), the Ni_r-B state of the immobilized enzyme is exclusively reduced (Ni^{3+}/Ni^{2+}) by the electrons delivered from the electrode through the FeS cluster chain (see SI 3A in the Supporting Information).[9] However, under an H_2 atmosphere (turnover), reduction may be achieved also by H_2 as suggested by Armstrong et al. (see SI 3B in the Supporting Information).[17] Different intermediates that may help to discriminate between these two reaction mechanisms (electrochemical or involving the substrate) have not yet been observed, and the only intermediate detected by IR spectroscopy (Ni_r-S) is involved in both reaction schemes. In a recent study on the [NiFe] hydrogenase from *Aquifex aeolicus*, Fourmond et al. demonstrated a linear dependence of E_{switch} with the logarithm of the scan rate v.[16] This behavior, which is consistent with our observations for the *Dv*MF hydrogenase implies that a comparison between $E_{1/2}$ and E_{switch} would be meaningful if measurements on the same time-scale are compared.[16] Thus, we have carried out potential-controlled experiments under 1 bar H_2 atmosphere by probing the immobilized *Dv*MF hydrogenase (see SI 4 in the Supporting Information) simultaneously by SEIRA spectroscopy and chronoamperometry (CA). The potential-dependent changes in the SEIRA spectra are qualitatively similar to those observed under non-turnover conditions, where the Ni_a-S contribution increases on the expense of the Ni_r-B state upon lowering the potential (see SI 5). This finding provides the first experimental evidence of the involvement of Ni_r-B and Ni_a-S in the reductive reactivation of an immobilized hydrogenase. The inflection point of the sigmoid-shaped curve obtained from the quantitative spectra analysis (see SI 6) corresponds to an E_{switch} value, which is identical to the value obtained from the concomitant chronoamperometric measurements.

There is an excellent agreement between electrochemical and spectroscopic measurements (see Table 1), supporting the high quality of the electrochemical control achieved with our setup. Furthermore, the value for E_{switch} is found to be more positive by approximately 50 mV than $E_{1/2}$ (see Table 1), which is in agreement with the estimation of Fourmond et al.[16] This difference, obtained by measurements on the same electrode sample under similar experimental conditions (pH value, temperature), may be due to the presence of H_2 as an additional reducing agent, as well as the deviation from the thermodynamic equilibrium related to the presence of the substrate. Fourmond et al. have predicted $E_{switch} > E_{1/2}$ for low scan rates although $E_{1/2}$ could not be determined directly for the immobilized enzyme.

In summary, we conducted the first SEIRA spectro-electrochemical analysis of an immobilized [NiFe] hydrogenase under turnover and non-turnover conditions. We have demonstrated that the Ni_r-B/Ni_a-S redox transition of the immobilized enzyme in the absence of H_2 is characterized by the same $E_{1/2}$ value as in solution-assayed experiments, revealing the integrity of the active-site structure in the adsorbed state and a good electrical communication with the SAM-coated Au electrode. In addition, under H_2 gas atmosphere, the E_{switch} value related to the Ni_r-B/Ni_a-S activation obtained concurrently by SEIRA spectroscopy and chronoamperometric measurements is higher than $E_{1/2}$, which is consistent with a recent theoretical analysis of E_{switch}.[16] In a broader perspective, the present study shows that SEIRA spectro-electrochemistry allows the assignment of redox transitions to individual species on the basis of their characteristic vibrational signatures. In this way, such an approach may substantially contribute to the elucidation of biocatalytic mechanisms of immobilized enzymes.

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