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Orientation and Function of a Membrane-Bound Enzyme Monitored by Electrochemical Surface-Enhanced Infrared Absorption Spectroscopy

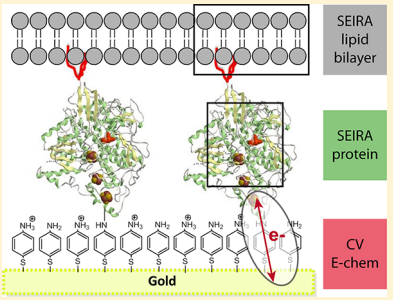
Oscar Gutiérrez-Sanz,[‡] Marta Marques,[§] Inês A. C. Pereira,[§] Antonio L. De Lacey,[‡] Wolfgang Lubitz,[†] and Olaf Rüdiger*[,†]

[†]Max-Planck-Institut für Chemische Energiekonversion, Stiftstrasse 34-36, 45470 Mülheim an der Ruhr, Germany

[‡]Instituto de Catálisis y Petroleoquímica, CSIC, c/Marie Curie 2, 28049 Madrid, Spain

[§]Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa Oeiras, Portugal

ABSTRACT: Electrochemical SEIRA is the method of choice for characterizing a lipid-bilayer-anchored, membrane-bound hydrogenase immobilized on a gold electrode. This setup allows the study of the enzyme under conditions mimicking its natural environment. A single experiment provides all of the crucial spectroscopic information relating to the protein orientation, active site of the protein, and the lipid bilayer and also direct electrochemical determination of the catalytic H_2 oxidation by the enzyme.



SECTION: Biophysical Chemistry and Biomolecules

Integration of redox proteins into nanostructured systems is of great interest for their application in biosensors, biofuel cells, nanobioelectronic devices, and nanobiocatalytic processes.[1] In this context, current research has focused on developing suitable interfaces able to link chemical reactions catalyzed by redox enzymes to electrochemical processes.[2] The goal is to optimize the immobilization of the enzyme on the conductive support while maintaining the biocatalyst's native structure and function and at the same time assuring the charge transport between both elements of the biodevice. This aim is especially challenging for membrane proteins due to their higher structural complexity and instability in the absence of their native lipid environment.[3] To deal with this problem several strategies have been developed for building biomimetic membranes on conductive supports.[4] Furthermore, these supported model membranes allow the study of membrane protein function.[5] One of the most useful techniques for studying the combination of membrane redox enzymes with biomimetic membranes supported on conductive surfaces is surface-enhanced infrared absorption (SEIRA) spectroelectrochemistry.[6−8] This technique allows the sensitive characterization of the chemical structure at the electrode interface while driving the electrocatalytic activity of the membrane enzyme. Moreover, it allows monitoring the step-by-step immobilization of a redox enzyme on an electrode by monitoring difference spectra.[6,9]

In this work we have studied the chemical structure and function of a membrane-attached hydrogenase coimmobilized with a phospholipid bilayer on a gold electrode, by SEIRA spectroelectrochemistry. Hydrogenases are redox metalloenzymes (containing Ni and/or Fe) that catalyze the reversible oxidation of H_2 to protons.[10−12] The study of hydrogenases is currently one of the most interesting areas of science from both the fundamental and application point of view. This is due to the worldwide growing interest in using H_2 for energy-conversion processes; therefore, the study of hydrogenases is important for developing alternative electrocatalysts to Pt in H_2 energy conversion technology. We have shown in a previous combined electrochemical and atomic force microscopy (AFM) study that the membrane-bound hydrogenase from *Desulfovibrio vulgaris* Hildenborough can be immobilized onto gold electrodes with its lipid tail inserted into a supported phospholipid bilayer.[13]

Moreover, the orientation of the enzyme relative to the electrode surface can be controlled by the immobilization procedure, either by inserting the hydrogenase on top of the phospholipid bilayer supported on the electrode or by forming the phospholipid bilayer on top of a hydrogenase monolayer covalently bound to the electrode (Figure 1). In the absence of phospholipids or detergents, the lipidic group located at the N terminus of the protein can be spontaneously cleaved, decreasing the catalytic activity of the resulting soluble enzyme. This suggested that the lipidic tail plays an important structural role in catalysis.[14] In the present work, for the first time we present data of the chemical structure of a membrane-bound hydrogenase immobilized at the electrode surface for both

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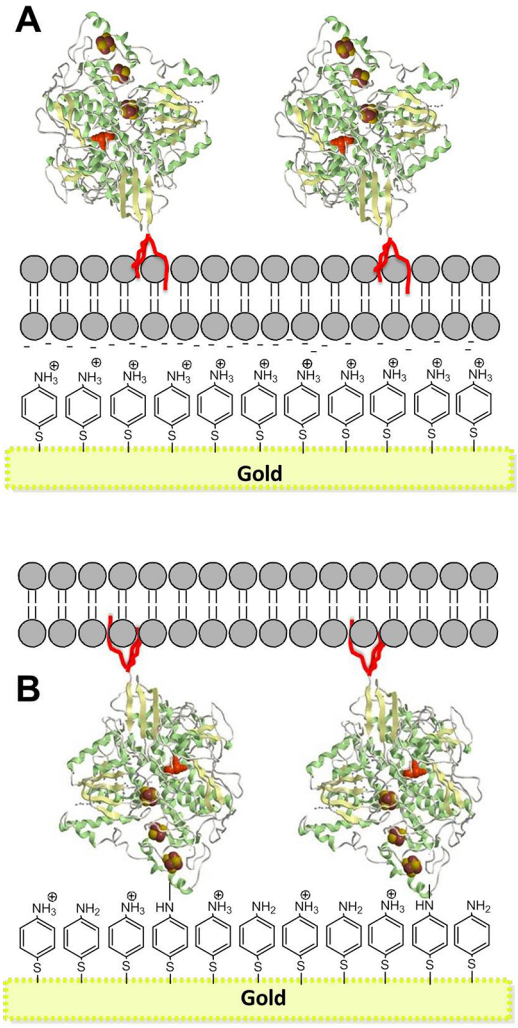

Figure 1. Schematic representation of the two immobilization strategies used. (A) Phospholipid bilayer is immobilized on top of a 4-aminothiophenol (4-ATP) self-assembled monolayer (SAM) and the protein is anchored afterward through the lipid tail (represented in red). (B) Protein is covalently immobilized on the 4-ATP SAM, and the lipid bilayer is formed afterward on top of the protein. Active site of the protein is indicated in orange and the [4Fe-4S] clusters are in yellow/brown.

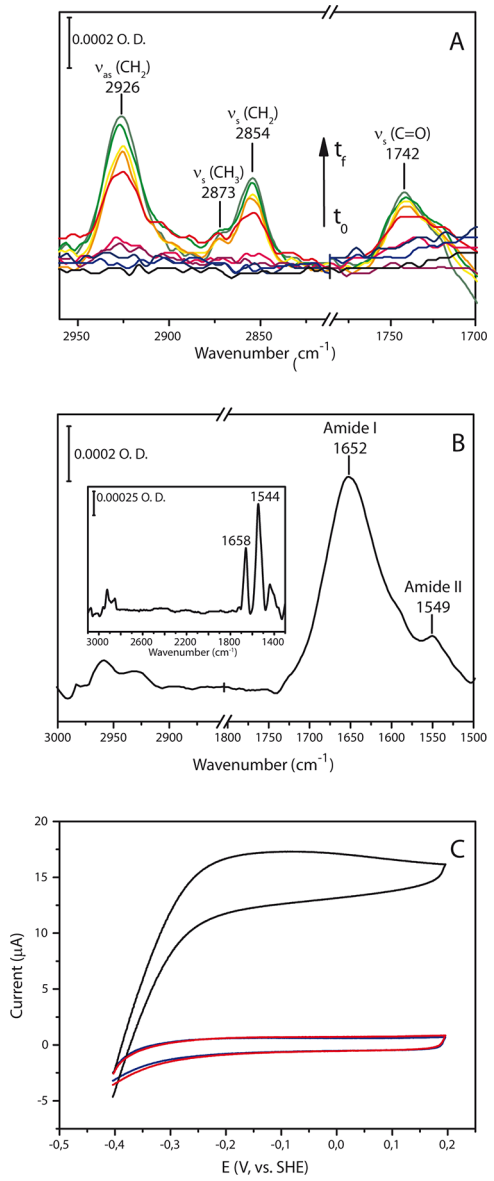

Figure 2. Results obtained with an electrode prepared with the protein immobilized on top of a phospholipid membrane (as represented in Figure 1A). (A) SEIRA spectra recorded during lipid layer formation on top of a 4-ATP SAM in MES buffer 10 mM at pH 5.0 over 10 h at 25 °C (black first spectrum, green final one). (B) SEIRA spectra after protein immobilization. The position of amide I and II bands is indicated. The inset corresponds to a SEIRA spectrum from a different electrode, prepared and measured under the same conditions. (C) Cyclic voltammograms (CVs) recorded after activation with H_2 without redox mediators in a N_2 (blue) or H_2 (red) atmosphere and after the addition of methyl viologen (MV) as a mediator (black).

orientations and correlate them with its in situ electrocatalytic activity for H_2 oxidation. We used SEIRA spectroscopy to study the step-by-step formation of the configuration shown in Figure 1A. The SEIRA spectra recorded at different times after the deposition of phospholipid vesicles from *Escherichia coli* polar fraction on a gold electrode modified with a 4-ATP monolayer are shown in Figure 2A. The reference spectrum for this experiment corresponds to the 4-ATP-modified gold surface. The appearance of vibrational bands at 2926, 2873, 2854, and 1742 cm^{-1} that increased during the time period of 10 h after the deposition of the vesicles is in agreement with the formation of a phospholipid bilayer,[6,8] as observed by AFM in a previous work.[13] The broad band at 2926 cm^{-1} is assigned to the asymmetrical stretching C−H modes of the phospholipids alkyl chains, whereas the 2873 and 2854 cm^{-1} bands are assigned to the symmetrical modes of the methyl and methylene groups, respectively. The band at 1742 cm^{-1} corresponds without doubt to the ester groups of the phospholipids forming the supported bilayer, as previously observed.[6,8]

After the formation of the supported phospholipid layer, a solution of detergent solubilized membrane-bound hydrogenase was deposited on top, in the presence of CALBIOSORB adsorbent, which consists of patented millispheres able to adsorb the detergent present in the hydrogenase solution without affecting the phospholipid bilayer.[13] The difference spectra of Figure 2B, using now as reference spectrum that of the phospholipid-modified electrode, shows the appearance of the typical amide I (at 1652 cm^{-1}) and amide II (at 1549 cm^{-1})

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vibrational bands of proteins.[15] These bands can only correspond to immobilized enzyme because SEIRA spectroscopy is not sensitive to distances beyond 10 nm from the metal surface.[16] The thickness of the phospholipid bilayer formed on the gold electrode modified with a 4-ATP SAM is approximately 4 to 5 nm, as previously determined by AFM,[13] and thus the SEIRA spectrum shows the insertion of the hydrogenase into the supported biomimetic membrane. The wavenumbers of these bands suggest that α-helices predominate in the secondary structure of the hydrogenase,[15] in agreement with its known crystal structure.[17] The high amide I/amide II intensity ratio in the spectrum indicates that most of these helices have a perpendicular orientation relative to the electrode surface, in this case.[18] However, this intensity ratio changed greatly between different experiments, performed under similar conditions (inset in Figure 2B). Changes of the amide I/amide II intensity ratio that were attributed to enzyme reorientation have also been reported for a hydrogenase adsorbed on a SAM-modified gold surface.[19,20] This suggests that there is considerable degree of freedom in the orientation of the hydrogenase molecules relative to the surface normal when they are immobilized through insertion of their lipid tail into the supported phospholipid bilayer.

Electrochemical measurements performed in the SEIRA cell showed that the hydrogenase inserted into the supported biomimetic membrane is catalytically active for H_2-oxidation, although only by mediated electron transfer using methyl viologen (MV) for transferring electrons from the enzyme to the electrode (Figure 2C). This electrocatalytic behavior is expected for the hydrogenase immobilized through its lipid tail because it will have its most exposed redox site (a [4Fe4S] cluster) to the solution and thus too far away from the electrode surface for establishing direct electron transfer.[13] MV added in solution is able to receive electrons from the hydrogenase and to donate them to the electrode by diffusing through defects in the phospholipid bilayer.[13]

SEIRA spectroscopy was also used for studying the step-by-step formation of the configuration shown in Figure 1B. In this configuration the hydrogenase is covalently immobilized to the 4-ATP-modified gold surface through amide bonds and with its lipid tail inserted into the phospholipid bilayer formed on top.[13] First, the covalent immobilization of the enzyme to the electrode was studied. Figure 3A shows the FTIR spectra recorded once the immobilization process is finished. The infrared spectrum shows the amide I and amide II vibrational bands of the immobilized hydrogenase at similar frequencies as before, but now the intensity ratio between these two bands is closer to 1. This effect indicates that the α-helices of the enzyme deviate now more from the gold surface normal on average,[18] which is in agreement with a different orientation of the immobilized hydrogenase in this configuration. In contrast with the previous case, this intensity ratio was reproducible in different experiments. This is expected for a more rigid immobilization of the hydrogenase molecules by covalent bonds to the electrode surface and with their orientation controlled by electrostatic interactions.[21] Furthermore, the vibrational bands due to the CO and CN^- ligands of the hydrogenase active site can be now detected (inset of Figure 3A), which was not the case for an electrode prepared as shown in Figure 1A. This is probably due to the shorter distance between the gold surface and the hydrogenase active site when it is attached directly to the gold surface. This increases the SEIRA effect and the spectral sensitivity. The frequencies of the

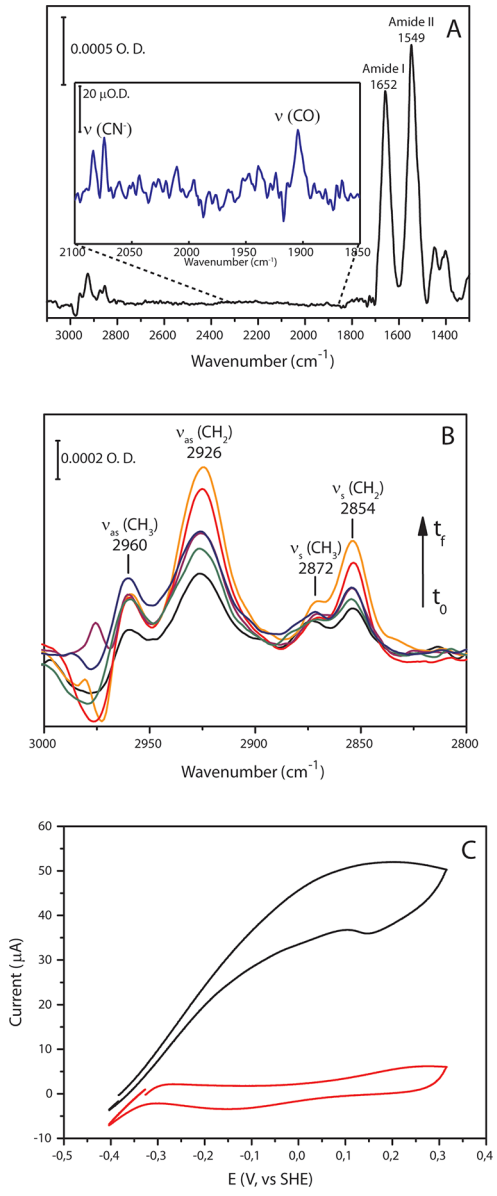

Figure 3. Results obtained with an electrode prepared with the protein immobilized on a 4-ATP SAM and the phospholipid membrane formed on top of the hydrogenase layer (as in Figure 1B). (A) SEIRA spectra after protein immobilization on a 4-ATP SAM, showing amide I and II bands. The inset shows in detail the active site region (ν(CO) 1906 cm^{-1}; ν(CN^-) 2072, 2085 cm^{-1}). (B) SEIRA spectra recorded during lipid layer formation on top of the hydrogenase in MES buffer 10 mM at pH 5.0 over 10 h (black first spectrum, orange final one). (C) CVs recorded after activation with H_2 (black) and in a N_2 atmosphere (red) in the absence of redox mediators. All measurements were performed at 25 °C.

CO band (1906 cm^{-1}) and the CN-bands (2072 and 2085 cm^{-1}) are very similar to those measured by transmission infrared spectroscopy for a solution of the same hydrogenase in the oxidized state (1904, 2076, and 2085 cm^{-1}).[22]

The SEIRA spectra recorded at different times after the deposition of phospholipid vesicles on top of the 4-ATP-hydrogenase-modified surface are shown in Figure 3B. When the vesicles were deposited on the modified gold surface, the vibrational bands of the C−H modes of the phospholipids alkyl chains gradually increased during the incubation time. This confirms that the topology change observed in the same system

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by AFM[13] corresponds to the formation of a phospholipid bilayer on top of the immobilized hydrogenase. The electrochemical measurements performed in the SEIRA spectroelectrochemical cell clearly show that in this configuration the hydrogenase can directly transfer electrons to the gold electrode and a H_2-oxidation catalytic current could be measured (Figure 3C). This electrocatalytic behavior is in agreement with the covalently bound hydrogenase having the orientation shown in Figure 1B, in which the [4Fe4S] cluster is facing the electrode and the opposite lipid tail is inserted into the phospholipid bilayer.

In conclusion, we have shown that electrochemical SEIRA can provide direct evidence of the insertion of a membrane-bound hydrogenase in a phospholipid bilayer. Moreover, it is shown that the protein acquires different orientations when the protein is electrostatically oriented by the SAM or when the orientation is provided by the lipid bilayer. These different orientations are corroborated by SEIRA. This strategy opens a new way to explore membrane-bound hydrogenases combining electrocatalytic and spectroscopic information in a single experiment using SEIRA.

EXPERIMENTAL SECTION

DvH [NiFeSe] hydrogenase was purified and biochemically characterized as described elsewhere.[14] SEIRA measurements were performed in the Kretschmann-ATR configuration.[6] Au thin films were formed on top of the Si prism by chemical deposition.[23] Suspension of liposomes for lipid bilayer formation were prepared and deposited on the electrodes from *E. coli* polar fraction, as previously described.[13] SEIRA spectra were recorded from 4000 to 1000 cm^{-1} with a spectral resolution of 2 cm^{-1} and accumulating 620 scans for each measurement on a Bruker Tensor 27 spectrometer equipped with an MCT detector. The gold electrode was modified with the 4-ATP SAM by the optimized conditions previously reported for measuring the electrocatalytic current of H_2 oxidation due to immobilized hydrogenase.[21] The hydrogenase and the phospholipid bilayer were immobilized on the 4-ATP-modified electrode by the two methods reported by Gutierrez-Sanchez et al.[13] The immobilization process was finished when the intensity of amide I/II bands did not increase any more over the time. Electrochemical measurements were controlled by a VersaStat 4-400 potentiostat (Princeton Applied Research).

AUTHOR INFORMATION

Corresponding Author

*E-mail: olaf.ruediger@cec.mpg.de.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AFM, atomic force microscopy; 4-ATP, 4-aminothiophenol; ATR, attenuated total reflection; CV, cyclic voltammetry; DvH, *Desulfovibrio vulgaris* Hildenborough; MCT, mercury cadmium telluride; MV, methyl viologen; SEIRA, surface enhanced infrared absorption spectroscopy

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