A Proton Gradient Across a Gold Supported Biomimetic Membrane Induced by Electro-Enzymatic H₂ Oxidation

Óscar Gutiérrez-Sanz ^[a], Cristina Tapia ^[a], Marta C. Marques ^[b], Sonia Zacarias ^[b], Marisela Vélez ^[a], Inês A. C. Pereira ^[b] and Antonio L. De Lacey ^{[a]*}

Dedicated to Prof. V. M. Fernandez on his 70th birthday

Abstract: Energy transduction mechanisms in living organisms, such as photosynthesis and respiration, store light and chemical energy in the form of an electrochemical gradient formed across a lipid bilayer. In this work we show that the proton concentration in an electrode/phospholipid bilayer interface can be controlled and monitored electrochemically by immobilizing a membrane-bound hydrogenase. Thus, the energy from electro-enzymatic oxidation of H₂ can be used to generate a proton gradient across the supported biomimetic membrane.

In many cases human society has progressed technologically by observing nature and copying its strategies, developed during millions of years of evolution. In the last years enzymes have inspired chemists for the development of new catalysts due to their specificity and high turnover in mild conditions.^[1] Furthermore, many industrial processes use biocatalytic routes based on enzyme activity.^[2] In a similar way, the field of bionanoelectronics has emerged for interfacing biological systems with artificial electronic structures aiming to establish communication between them in both directions.^[3] The combination of biology, electrochemistry and nanotechnology provides potential alternative and innovative solutions to face challenges in various fields (i.e. medicine, analytical chemistry, alternative energies, new materials development).

Energy transduction mechanisms in living organisms, such as photosynthesis and respiration, store light and chemical energy in the form of an electrochemical gradient formed across a lipid bilayer, as described by the chemiosmotic theory proposed by Mitchell.^[4] Model membranes can be stably formed over conductor surfaces for studying biological systems and potential biotechnological applications.^[5] Adequate tailoring of the conductor surface and biomimetic membrane formation allows the incorporation of membrane-bound enzymes maintaining their functionality.^[6]

In this work we show that a modified surface, in which a hydrogenase is immobilized between a phospholipid bilayer and a gold electrode, permits to store the energy produced by electrochemically driven H₂ consumption in a proton gradient across the supported membrane that can be monitored electrochemically. For this purpose we used two strategies developed in our laboratory: (a) the oriented and functional immobilization of the membrane-bound NiFeSe hydrogenase from *Desulfovibrio vulgaris* Hildenborough (Dv-SeHase) onto a gold electrode with a phospholipid bilayer (PhLB) on top^[7] and (b) the monitoring of the proton concentration in a phospholipid bilayer/electrode interface by an immobilized redox probe.^[8]

[a] O. Gutiérrez-Sanz, C. Tapia, Dr. M. Vélez, Dr. A. L. De Lacey
Instituto de Catálisis y Petroleoquímica
CSIC
c/ Marie Curie 2, 28049 Madrid, Spain
E-mail: alopez@icp.csic.es

[b] M. C. Marques, S. Zacarias, Dr. I. A. C. Pereira
Instituto de Tecnologia Química e Biológica,
Universidade Nova de Lisboa
Apartado 127,2781-901 Oeiras, Portugal

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Figure 1. Schematic representation of Dv-SeHase immobilized covalently to a Au electrode modified with a SAM of 4-ATP and oriented with its lipidic tail inserted into a phospholipid bilayer (Au/4-ATP/Dv-SeHase/PhBL), in accordance with previous work [7]. The lower panel shows sequential cyclic voltammograms (CV) at 0.1 V/s performed with the Au/4-ATP electrode (in order to obtain a pH-dependent redox couple). The black solid line shows the first one and the red dashed line the 15th of the series. The inset shows various Differential Pulse Voltammetry (DPV) measurements carried in phosphate buffer 0.1M with different pH values (from left to the right 8.0, 7.0, 6.2 and 5.5). Modulation time was 0.05 s, interval time was 0.1 s and modulation amplitude was 0.01 V.)

The structural and catalytic characteristics of the Dv-SeHase make this enzyme ideal for our purpose. Firstly, this hydrogenase has a lipid tail in the opposite region to the distal iron-sulphur cluster ([4Fe4S]),^[9] which is the redox site for electron transfer with the electrode. The distal [4Fe4S] cluster is surrounded by negatively charged aminoacids that allow the enzyme orientation by electrostatic interactions with the partially protonated self-assembled monolayer (SAM) of 4-aminothiophenol (4-ATP) on the electrode followed by covalent binding,^[10] and the lipid tail allows formation of a biomimetic bilayer on top.^[7] Figure 1 is a schematic representation of the configuration of this biomimetic construction. Secondly, like hydrogenases, Dv-SeHase catalyzes reversibly the oxidation of molecular hydrogen to protons. In particular, NiFeSe-hydrogenases have been shown to be tolerant to the presence of O₂ (a common inhibitor of many hydrogenases) during H₂-production activity when immobilized on electrodes or semiconductors.^[11]

Figure 2 shows a chronoamperometric measurement of the H₂-production activity of the Au/4-ATP/Dv-SeHase/PhBL electrode. A stable cathodic current is measured at -340 mV due to direct electron transfer to the enzyme and its proton reduction activity^[10]. Upon addition of 20 μM O₂, an immediate increase of the negative current is observed, due to its direct reduction at the electrode,^[11a] but after 5-6 minutes the initial catalytic current level of H₂-production is recovered due to linear diffusion limitation of oxygen transport towards the electrode and to equilibration of the solution with the N₂ atmosphere. Subsequent addition of 20 μM CO (also a known inhibitor of hydrogenases)^[12] abolishes completely the hydrogenase catalytic activity and the reductive current is suppressed. These results confirm that the immobilized hydrogenase does not become inactivated by

O₂ under H₂-production conditions, which can be explained by the favoured reaction of selenocysteines with O₂ to form a selenoate that its quickly reversed at low redox potential.^[13] Moreover, the addition of a similar O₂ aliquot, after the complete inhibition with CO, induces the recovery of the initial current of H₂ production (Figure 2). This electrochemical behaviour is similar to that reported for the soluble NiFeSe hydrogenase from *D. Baculatum*,^[11a] and FTIR measurements of the Dv-SeHase have shown that extrinsic CO bound to its active site is removed by O₂.^[14]

Figure 2: Chronoamperometry performed at -340 mV vs. SHE under N₂ atmosphere in 0.1M, pH 7.6 phosphate buffer at 30°C with the Au/4-ATP/Dv-SeHase/PhBL electrode. The arrows mark the additions of oxygen and carbon monoxide. The straight solid line indicates the stable catalytic current of the enzymatic H₂-production activity, and the dashed straight line marks the background current with inhibited hydrogenase.

After showing that the Au/4-ATP/Dv-SeHase/PhBL electrode has a stable electrocatalytic activity that is not affected irreversibly by the CO and O₂ inhibitors, we studied the generation of a proton gradient across the biomimetic membrane. The oxidation of the 4-ATP SAM on the gold electrode generates a pH-dependent redox couple, as shown in the cyclic voltammograms (CVs) and differential pulse voltammograms (DPV) of Fig. 1B.^[15] The measured shift of the peak potential of the DPV is 64 ± 4 mV per pH unit (Figure 3), which is near the theoretical value for a $2e^-/2H^+$ at 30°C (60 mV).^[16] Thus, this redox process has been used in our system to probe the pH changes at the electrode/bilayer interface.

Figure 3: Calibration line obtained from DPV measurements at different pH values in 0.1 M phosphate buffer and 30°C with a Au/4-ATP electrode after the oxidative treatment. The equation of the fitted line is shown in the graph.

In order to obtain the redox probe signal for the Au/4-ATP/Dv-SeHase/PhLB electrode, 15 CVs were scanned between 0.25 and 0.70 V. The first DPV that was measured after the oxidative treatment of the electrode presented an oxidation peak at 380 mV (Figure 4, solid line). Afterwards, the immobilized hydrogenase was activated during 30 minutes under an H₂ atmosphere (Figure S1a). A DPV measurement was carried out with a pre-treatment of 90 s of electro-enzymatic oxidation of H₂ at 640 mV, followed by a resting time of 60 seconds, in which 25 μ M CO was injected into the solution. The addition of CO was required to suppress the electrocatalytic current, allowing the clear detection of the DPV peak at 493 mV (Figure 4, dashed line). The peak potential shift after the period of H₂-oxidation activity corresponds to a change of nearly 2 pH units at the electrode boundary, which means that the hydrogenase activity accumulated protons in the electrode/bilayer interface. Finally, O₂ was added to the solution to remove the CO inhibiting the hydrogenase active site and a reduction potential of -340 mV under N₂ atmosphere was applied until the catalytic current of H₂-production was established (Figure 2). After 15 minutes in these conditions, a new DPV measurement was recorded. This time, the enzyme was not inhibited with CO for the DPV measurement because under N₂ atmosphere the electrocatalytic current at positive potentials is negligible,^[10, 14] thus there is no interference for measuring the redox probe oxidation peak by DPV (Figure 4, dotted line). The pH value of the electrode/PhLB interface recovered its initial value due to proton reduction catalysed by the hydrogenase. The last DPV peak was broader than the previous ones, suggesting that there is some heterogeneity in the interface conditions in different regions of the modified electrode, although it is clear that in general the amount of protons had increased. Therefore, the pH gradient generation across the biomimetic membrane by the hydrogenase was observed to be reversible: H₂-oxidation activity shifted the pH at the electrode/PhBL interface to more acidic values and H₂-production activity shifted it to more alkaline values. It was checked with a pH-meter that the bulk buffered solution of the electrochemical cell did not change its pH during the experiment. The enzymatic electrode response was stable at least during 10 successive experiments of H₂-oxidation/H⁺-reduction cycles. Longer operational stability was not studied.

Figure 4: DPV measurements of the Au/4-ATP/Dv-SeHase/PhLB electrode in pH 8, 0.1 M phosphate buffer at 30°C under N₂ atmosphere before hydrogenase activation (solid line), after electro-enzymatic H₂-oxidation (dashed line) and after electro-enzymatic H₂-production under N₂ atmosphere (dotted line).

A control experiment without PhBL formation over the Au/4-ATP/Dv-SeHase electrode was performed to demonstrate that the change of the proton concentration at the electrode boundary monitored in the experiments of Figure 4 requires the presence of an electrode/PhLB interface. No shift of pH at the electrode boundary was observed after doing DPV measurements under N₂ or H₂ atmosphere in absence of the supported PhBL (Figure 5a), even if the immobilized hydrogenase was catalytically active (Figure S1b). The control measurement in absence of enzyme was also done with a Au/4-ATP/PhLB electrode (Figure 5b), confirming that the pH gradient was only generated in presence of active Dv-SeHase in the electrode/bilayer interface.

Figure 5: DPV measurements with a Au/4-ATP/Dv-SeHase electrode (a) and Au/4-ATP/PhLB electrode (b) in phosphate buffer 0.1M pH 8 at 30°C under N₂ atmosphere before enzyme activation (solid line), or after electro-enzymatic of H₂-oxidation (dashed line).

In conclusion, this work presents electrochemical evidence that H₂ consumption by a membrane-bound hydrogenase on a modified gold electrode generates a proton gradient. This method for monitoring and controlling the membrane potential in bioelectronic devices could be used to store energy produced from H₂ for several applications, such as artificial ATP production, drug testing in biomimetic environment, development of new biosensors, as well as for fundamental studies of cell metabolism. Future work aims the coupling of the developed system to the activity of an ATP-synthase.

Experimental Section

Dv-SeHase was isolated and purified as reported previously.^[18] 0.2 mg/mL liposomes suspension, Au electrodes preparation and modification with 4-ATP were done as described by Gutierrez-Sanz *et al.*^[8] Au/4-ATP/Dv-SeHase/PhBL electrodes were constructed by incubating Au/4-ATP electrodes during 90 minutes in a 12 μM Dv-SeHase solution in pH 5.5, 0.1 M phosphate buffer 0.1 M and 0.1% N-Dodecyl β-D-maltoside (DDM) at 4°C. Then, the electrodes were incubated for 30 minutes in 21 mM N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride in pH 6.0, 50 mM Tris-HCl buffer with 0.1% DDM. Finally, the electrodes were incubated during 90 minutes in a 0.2 mg/ml liposomes suspension in presence of 240 mg/mL CALBIOSORB adsorbent biobeads (Calbiochem). Electrochemical experiments were done as reported previously.^[7a] Au/4-ATP/PhBL electrodes were prepared as described by Gutierrez-Sanz *et al.*^[8]

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