Enzymatic Electrodes

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Induction of a Proton Gradient across a Gold-Supported Biomimetic Membrane by Electroenzymatic H_2 Oxidation**

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Dedicated to Professor V. M. Fernandez on the occasion of 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 created across a lipid bilayer. Herein we show that the proton concentration at an electrode/phospholipid-bilayer interface can be controlled and monitored electrochemically by immobilizing a membrane-bound hydrogenase. Thus, the energy derived from the electroenzymatic oxidation of H_2 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, which have developed during millions of years of evolution. In the last two decades, the specificity and high turnover of enzymes under mild conditions has inspired the development of new catalysts.[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 with the aim of establishing communication between them in both directions.[3] The combination of biology, electrochemistry, and nanotechnology provides potential alternative and innovative solutions to the challenges in various fields (i.e. medicine, analytical chemistry, alternative energies, 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 created across a lipid bilayer, as described by the chemiosmotic theory proposed by Mitchell and Moyle.[4] Model membranes can be stably formed over conductor surfaces for the study of biological systems and potential biotechnological applications.[5] Adequate tailoring of the conductor surface and biomimetic membrane formation enables the incorporation of membrane-bound enzymes that maintain their functionality.[6]

Herein we show that a modified surface, in which a hydrogenase is immobilized between a phospholipid bilayer and a gold electrode, permits the storage of energy produced by electrochemically driven H_2 consumption in a proton gradient across the supported membrane; this proton gradient 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 (PhBL) on top[7] and b) monitoring of the proton concentration at a phospholipid-bilayer/electrode interface by an immobilized redox probe.[8]

The structural and catalytic characteristics of the *Dv*-SeHase make this enzyme ideal for our purpose. First, this hydrogenase has a lipid tail in the opposite region to the distal iron–sulfur cluster ([4Fe4S]),[9] which is the redox site for electron transfer with the electrode. The distal [4Fe4S] cluster is surrounded by negatively charged amino acids that enable 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 enables the formation of a biomimetic bilayer on top.[7] Figure 1a is a schematic representation of the configuration of this biomimetic construction. Second, like hydrogenases, *Dv*-SeHase catalyzes reversibly the oxidation of molecular hydrogen to protons. In particular, NiFeSe hydrogenases have been shown to tolerate the presence of O_2 (a common inhibitor of many hydrogenases) during H_2-production activity when immobilized on electrodes or semiconductors.[11]

Figure 2 shows the chronoamperometric measurement of the H_2-production activity of the Au/4-ATP/*Dv*-SeHase/PhBL electrode. A stable cathodic current was measured at −340 mV owing to direct electron transfer to the enzyme and its proton-reduction activity.[10] Upon the addition of O_2 (20 μM), an immediate increase in the negative current was observed as a result of the direct reduction of O_2 at the electrode;[11a] however, after 5–6 min, the initial catalytic current level of H_2 production was recovered owing to the linear diffusion limitation of oxygen transport towards the electrode and to equilibration of the solution with the N_2 atmosphere. The subsequent addition of CO (20 μM; also

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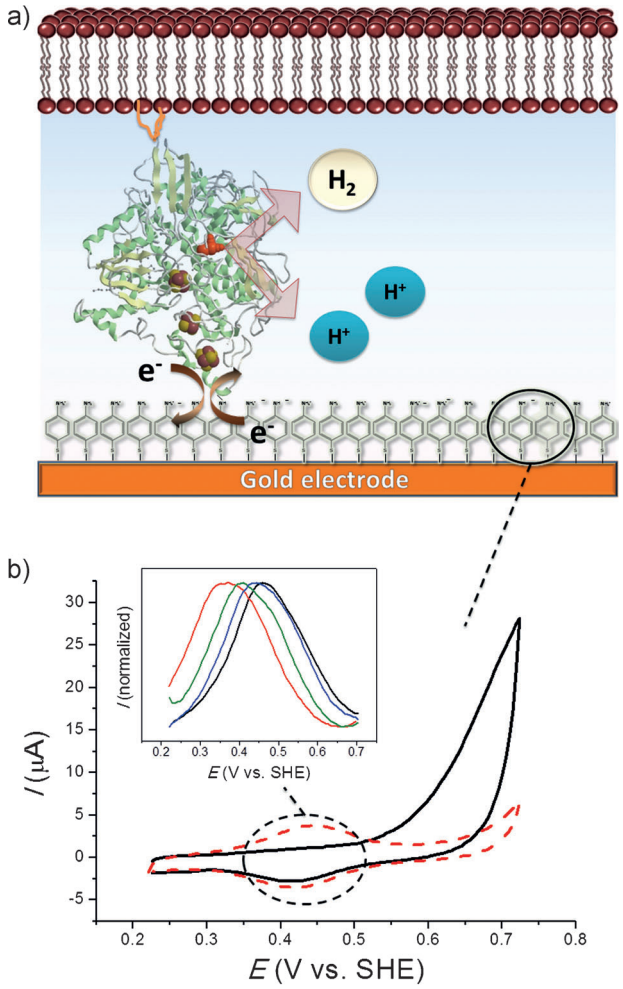

Figure 1. a) Schematic representation of *Dv*-SeHase immobilized covalently on a Au electrode modified with a SAM of 4-ATP and oriented with its lipidic tail inserted into a phospholipid bilayer (Au/4-ATP/*Dv*-SeHase/PhBL), in accordance with previous studies.[7] b) Sequential cyclic voltammograms (CVs) at 0.1 Vs^{-1} performed with the Au/4-ATP electrode (to obtain a pH-dependent redox couple). The black solid line shows the first and the red dashed line the 15th CV of the series. The inset shows various differential pulse voltammetry (DPV) measurements carried out in 0.1 M phosphate buffer with different pH values (from left to right: pH 8.0, 7.0, 6.2, and 5.5). The modulation time was 0.05 s, the interval time was 0.1 s, and the modulation amplitude was 0.01 V. SHE = standard hydrogen electrode.

a known inhibitor of hydrogenases)[12] abolished completely the hydrogenase catalytic activity, and the reductive current was suppressed. These results confirm that the immobilized hydrogenase does not become inactivated by O_2 under H_2-production conditions and can be explained by the favored reaction of selenocysteines with O_2 to form a selenoate and the rapid reversal of this reaction at low redox potential.[13] Moreover, the addition of a similar O_2 aliquot, after complete inhibition with CO, induced the recovery of the initial current of H_2 production (Figure 2). This electrochemical behavior is similar to that reported for the soluble NiFeSe hydrogenase from *Desulfomicrobium Baculatum*,[11a] and FTIR measurements of the *Dv*-SeHase have shown that extrinsic CO bound to its active site is removed by O_2.[14]

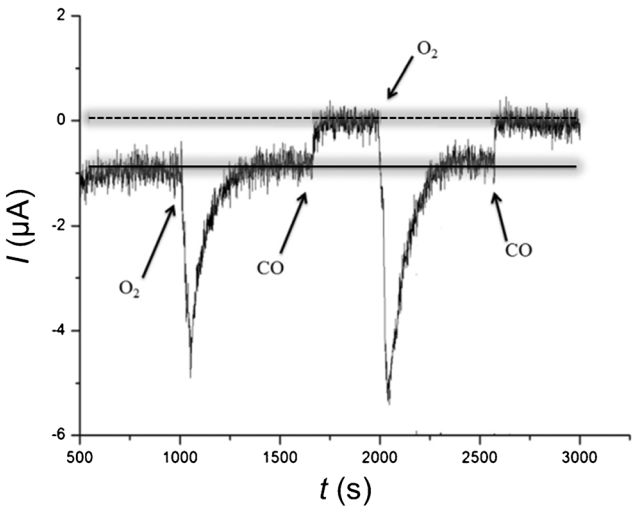

Figure 2. Chronoamperometry performed at −340 mV vs. SHE under a N_2 atmosphere in 0.1 M phosphate buffer (pH 7.6) at 30 °C with the Au/4-ATP/*Dv*-SeHase/PhBL electrode. The arrows mark the addition of oxygen and carbon monoxide. The straight solid line indicates the stable catalytic current of the enzymatic H_2-production activity, and the dashed straight line marks the background current with inhibited hydrogenase.

After demonstrating that the Au/4-ATP/*Dv*-SeHase/PhBL electrode shows stable electrocatalytic activity that is not affected irreversibly by the CO and O_2 inhibitors, we studied the generation of a proton gradient across the biomimetic membrane. 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 (DPVs) in Figure 1b.[15] The measured shift of the DPV peak potential is (64 ± 4) mV per pH unit (Figure 3) and is thus near the theoretical value for $2e^-/2H^+$ at 30 °C (60 mV).[16] Therefore, this redox process was used in our system to probe the pH changes at the electrode/bilayer interface.

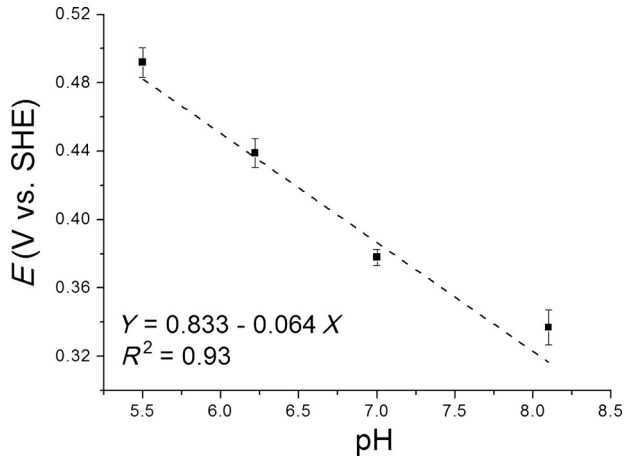

Figure 3. Calibration line obtained from DPV measurements at different pH values in 0.1 M phosphate buffer at 30 °C with a Au/4-ATP electrode after oxidative treatment. The equation of the fitted line is shown in the graph.

To obtain the redox-probe signal for the Au/4-ATP/*Dv*-SeHase/PhBL electrode, we recorded 15 CVs between 0.25 and 0.70 V. The first DPV that was measured after oxidative treatment of the electrode presented an oxidation peak at 380 mV (Figure 4, solid line). Afterwards, the immobilized hydrogenase was activated over a period of 30 min under an H_2 atmosphere (see Figure S1 b in the Supporting Information). A DPV measurement was made after pretreatment by

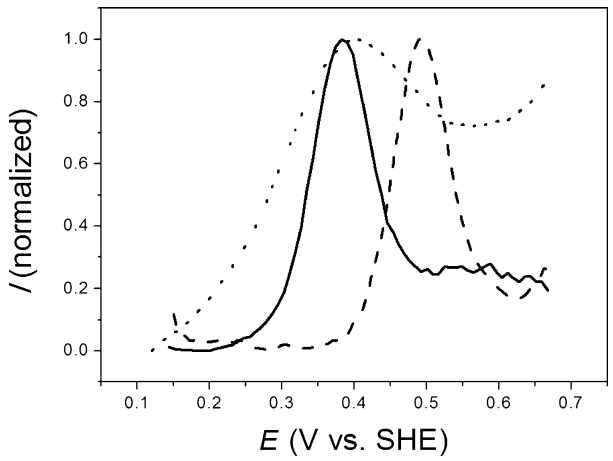

Figure 4. DPV measurements of the Au/4-ATP/*Dv*-SeHase/PhBL electrode in 0.1 M phosphate buffer (pH 8) at 30 °C under a N_2 atmosphere before hydrogenase activation (solid line), after electroenzymatic H_2 oxidation (dashed line), and after electroenzymatic H_2 production under a N_2 atmosphere (dotted line).

the electroenzymatic oxidation of H_2 at 640 mV for 90 s, followed by a resting time of 60 s, during which CO (25 μM) was injected into the solution. The addition of CO was required to suppress the electrocatalytic current, thus allowing the clear detection of the DPV peak at 493 mV (Figure 4, dashed line). The peak potential shift after the period of H_2-oxidation activity corresponds to a change of nearly 2 pH units at the electrode boundary, which means that the hydrogenase activity led to the accumulation of protons at the electrode/bilayer interface. Finally, O_2 was added to the solution to remove the CO inhibiting the hydrogenase active site, and a reduction potential of −340 mV was applied under a N_2 atmosphere until the catalytic current of H_2 production was established (Figure 2). After 15 min under these conditions, a new DPV measurement was recorded. This time, the enzyme was not inhibited with CO for the DPV measurement because under a N_2 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/PhBL interface recovered its initial value as a result of proton reduction catalyzed by the hydrogenase. The last DPV peak was broader than the previous DPV peaks, thus 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 decreased. Therefore, the generation of a pH gradient across the biomimetic membrane by the hydrogenase was observed to be reversible: H_2-oxidation activity shifted the pH value at the electrode/PhBL interface to more acidic values, and H_2-production activity shifted it to more alkaline values. We checked with a pH meter that the bulk buffered solution of the electrochemical cell did not change its pH value during the experiment. The enzymatic electrode response was stable at least during 10 successive experiments involving H_2-oxidation/H^+-reduction cycles. Longer operational stability was not studied.

A control experiment without PhBL formation over the Au/4-ATP/*Dv*-SeHase electrode was performed to demonstrate that the change in the proton concentration at the electrode boundary as monitored in the experiments of Figure 4 requires the presence of an electrode/PhBL interface. No shift in the pH value at the electrode boundary was observed in DPV measurements made under a N_2 or H_2 atmosphere in the absence of the supported PhBL (Figure 5 a), even if the immobilized hydrogenase was catalytically active (see Figure S1 a). A control measurement in the absence of the enzyme was also made with a Au/4-ATP/PhBL electrode (Figure 5 b) and confirmed that the pH gradient was only generated in the presence of active *Dv*-SeHase at the electrode/bilayer interface.

In conclusion, we have presented electrochemical evidence that H_2 consumption by a membrane-bound hydrogenase on a modified gold electrode generates a proton gradient. This method for monitoring and controlling the

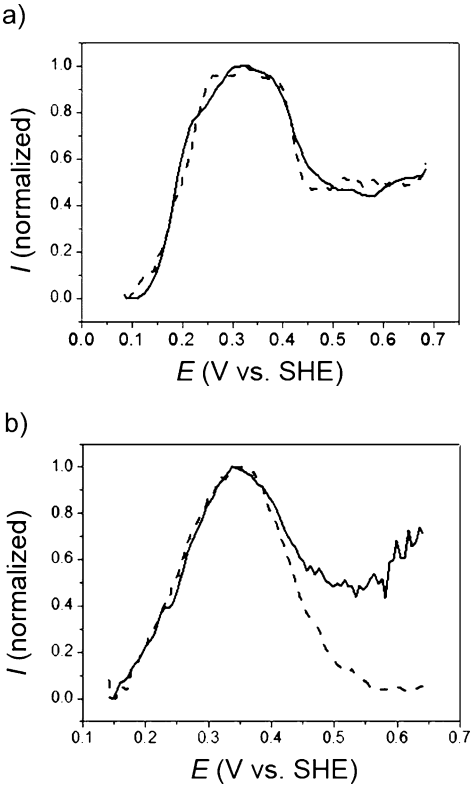

Figure 5. DPV measurements with a) a Au/4-ATP/*Dv*-SeHase electrode and b) a Au/4-ATP/PhBL electrode in 0.1 M phosphate buffer (pH 8) at 30 °C under a N_2 atmosphere before enzyme activation (solid line) and after electroenzymatic H_2 oxidation (dashed line).

membrane potential in bioelectronic devices could be used to store energy produced from H_2 for several applications, such as artificial adenosine triphosphate (ATP) production, drug testing in a biomimetic environment, and the development of new biosensors, as well as for fundamental studies of cell metabolism. Future studies aim to couple the developed system to the activity of an ATP synthase.

Experimental Section

Dv-SeHase was isolated and purified as reported previously.[17,18] A 0.2 mg mL^{-1} liposome suspension was prepared and Au electrodes were prepared and modified with 4-ATP as described previously.[8] Au/4-ATP/*Dv*-SeHase/PhBL electrodes were constructed by incubating Au/4-ATP electrodes for 90 min in a 12 μM solution of *Dv*-SeHase in 0.1M phosphate buffer (pH 5.5) with 0.1 % *n*-dodecyl-β-D-maltoside (DDM) at 4 °C. The electrodes were then incubated for 30 min in a 21 mM solution of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride in 50 mM Tris-HCl buffer (pH 6.0) with 0.1 % DDM. Finally, the electrodes were incubated for 90 min in a 0.2 mg mL^{-1} liposome suspension in the presence of 240 mg mL^{-1} Calbiosorb adsorbent biobeads (Calbiochem). Electrochemical experiments were carried out as reported previously.[7a] Au/4-ATP/PhBL electrodes were prepared as described previously.[8]

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