Communication

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Enzymatic Oxidation of H_2 in Atmospheric O_2: The Electrochemistry of Energy Generation from Trace H_2 by Aerobic Microorganisms

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A considerable number of microbes, such as *Ralstonia spp.*, are able to exploit H_2 as an energy source in the course of aerobic respiration, using enzymes known as hydrogenases to catalyze the oxidation of H_2.[1,2] The bimetallic active site of [NiFe]-hydrogenases comprises an Fe atom, coordinated by the biologically unusual ligands CO and CN^-, which is linked to a Ni atom by bridging cysteine ligands.[3] High turnover frequencies for H_2 oxidation, in excess of 1000 s^{-1}, have been reported for some [NiFe]-hydrogenases. Indeed, at 1 bar H_2, the rate of H_2 oxidation catalyzed by the membrane-bound hydrogenase (MBH) from *Allochromatium vinosum (Av)* is comparable to that of Pt operating under neutral, aqueous conditions.[4,5] This high activity has accelerated interest in hydrogenases or enzyme-inspired alternatives to Pt, as anode catalysts in fuel cells.

Hydrogenases are notoriously air-sensitive, and in most cases they are inactivated or destroyed by O_2 in vitro.[6] (As a small, uncharged molecule, O_2 is able to permeate cell membranes and atmospheric O_2 is therefore also deleterious in vivo.) For example, the MBH from the anoxygenic phototroph *Av* is completely inactive in H_2 containing just 0.5% O_2, and is slowly reactivated by reduction under anaerobic conditions. In contrast, under identical conditions, the MBH from the aerobic Knallgas bacterium *Ralstonia eutropha* H16 (*Re*) retains approximately 70% of its anaerobic H_2 oxidation activity.[7] The MBH from *R. metallidurans* CH34 (*Rm*), which is closely related to *Re* MBH, was recently used as the anode catalyst in a membraneless fuel cell operating on 3% H_2 in air.[8] However, to date, no energy-converting hydrogenase has been identified that retains full activity in air. Inhibition by O_2 is complex, and several states are implicated.[5] The structure of an O_2-tolerant hydrogenase has yet to be determined.

Measurement of H_2 oxidation activity in air is not possible using soluble electron mediators: low-potential acceptors would be oxidized by O_2, whereas high potential acceptors would inactivate the enzyme.[6] Direct electrochemical methods,[5] however, allow the potential to which the hydrogenase is exposed to be precisely controlled, permitting "fine-tuning" of the net reactions that are observed. The catalytic current is a direct measure of the rate of H_2 oxidation. We therefore pose the question: what level of H_2 can an O_2-tolerant hydrogenase oxidize in air?

In anaerobic wetland soil environments, H_2 is produced from organic substrates by fermentative bacteria and is consumed by methanogens and other microorganisms. This gives rise to steady-state H_2 levels in the range 10−180 ppm.[9] The H_2 concentration reaching the aerobic habitats of Knallgas bacteria is even lower.[9] At sea level, H_2 is present in the atmosphere at about 0.5 ppm.

We demonstrate here quantitative measurements of H_2 oxidation at extremely low, physiologically relevant levels of H_2, *in the presence of atmospheric levels of* O_2. We make use of a modified version of the method described recently by Léger et al.[10] for determining the affinities for various gases of an O_2-sensitive hydrogenase under anaerobic conditions.

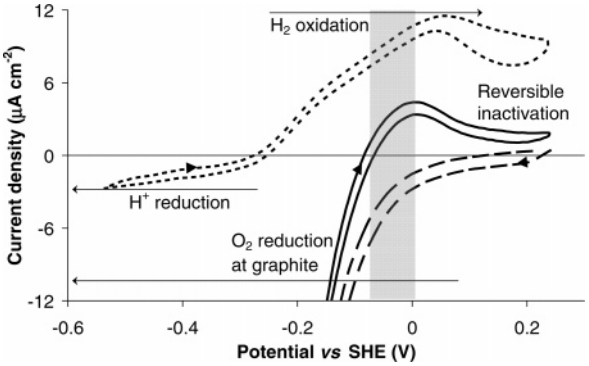

Figure 1. Cyclic voltammograms showing the current vs potential response for an electrode (area 0.03 cm^2) modified with *Rm* MBH under 1% H_2 in N_2 (dotted) and 1% H_2 in air (bold). Also shown is the response of a blank electrode under 1% H_2 in air (dashed). Conditions: pH 5.5, temperature = 30 °C, electrode rotation rate = 4500 rpm, scan rate = 2 mV s^{-1}. The gray bar indicates the potential window within which MBH-dependent H_2 oxidation in air can be measured reliably.

Figure 1 shows cyclic voltammograms recorded at a 0.03 cm^2 PGE electrode modified with *Rm* MBH under 1% H_2 in N_2 (dotted) and 1% H_2 in air (bold) at 30 °C.[11] Also shown is the response of an unmodified "blank" electrode in 1% H_2 in air (dashed).

In the anaerobic case, the oxidation current at potentials more positive than −280 mV vs SHE is due to electrocatalytic H_2 oxidation by the enzyme. At potentials more positive than about +80 mV reversible inactivation is observed.[6] In the aerobic case, direct reduction of O_2 at bare regions of the graphite electrode dominates the voltammogram at potentials more negative than −80 mV. Above this potential, the net current response is dominated by enzymatic H_2 oxidation, which again shows reversible inactivation. Under atmospheric O_2 levels there is therefore a window of potential (highlighted in Figure 1) in which H_2 oxidation activity can be monitored, minimizing the complications of O_2 reduction and enzyme inactivation. The smaller H_2 oxidation current in the aerobic scan relative to the anaerobic scan is caused by a partial inhibition of the enzyme, and also by competing O_2 reduction below 0 mV (see unmodified electrode trace, dashed).

Figure 2 shows an experiment in which H_2 oxidation activity is measured at varying levels of H_2 in air at 30 °C. An electrode (area 0.03 cm^2) is modified with *Rm* MBH and the H_2 oxidation activity is measured at constant potential (0 mV) as the H_2 concentration is decreased continuously from 79% by constant flow gas exchange with a carrier gas containing zero H_2. The headgas within the sealed glass cell is prepared and supplied using mass flow controllers

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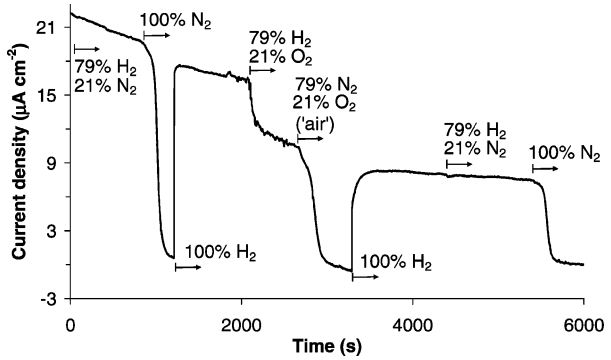

Figure 2. Current vs time response for an electrode (0.03 cm^2) modified with *Rm* MBH under varying gas atmospheres. Conditions: pH 5.5, temperature = 30 °C, electrode rotation rate = 4500 rpm, electrode potential = 0 mV vs SHE.

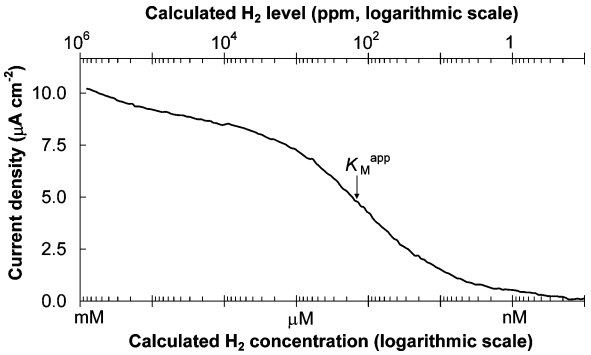

Figure 3. Transform of aerobic data from Figure 2 showing the current vs H_2 concentration trace for an electrode modified with *Rm* MBH. The arrow denotes the approximate value of K_M^{app} in the presence of 21% O_2.

(Sierra Instruments, accuracy ± 1%; typical flow rate 800 mL per minute) and the electrode is rotated at a high constant rate (4500 rpm) to ensure the H_2 concentration at the electrode surface remains close to that of the bulk solution. The experiment consists of a sequence of gas exchanges: first the H_2 was flushed out using N_2 as the carrier gas, H_2 was then reintroduced, and then it was flushed out with 21% O_2/79% N_2 (i.e., artificial air). Finally H_2 was reintroduced then flushed out with N_2. In agreement with the observations of Léger et al.,[10] removal of H_2 from the cell follows an exponential course over time (t) with a time constant τ (eq 1). The τ value is a function of several factors, all of which had to be kept constant, but by using this sequence of gas exchanges, we could verify τ did not change during the course of the experiment.

$$[H_2]_{(t)} = [H_2]_{(0)} \exp\left(\frac{-t}{\tau}\right) \quad (1)$$

Combining the Michaelis−Menten equation with eq 1 gives eq 2, allowing calculation of τ.[10]

$$\log_{10}\left(\frac{i_{max}}{i_t} - 1\right) = \log_{10}\left(\frac{K_M}{C_{H_2}(0)}\right) + \frac{t}{2.3\tau} \quad (2)$$

Each phase of gas exchange produces a sigmoidal decrease in current. The current is initially insensitive to the loss of H_2 while its concentration remains well above the Michaelis-Menten constant for H_2 oxidation, K_M.

Because the carrier gas flow rate remains constant throughout the experiment, the H_2 concentration dependence of the H_2 oxidation rate is obtained by substituting τ into eq 1. A typical result for the aerobic phase is shown in Figure 3.

Figure 3 shows that the H_2 oxidation activity is substantial (>50%) at 1 μM (1000 ppm) H_2 and is still detectable below 10 nM (10 ppm). The approximate value of an apparent K_M (K_M^{app}) is indicated, noting that interpreting K_M in the presence of O_2 is particularly problematic as O_2 is not a simple competitive inhibitor.[10] It is important also to note that although even at 0 mV a small degree of direct O_2 reduction may contribute to the current during the aerobic phase, this component remains constant throughout and does not contribute to the decrease. Even allowing for non-idealities such as slow film-loss, the conclusion is that H_2 oxidation under aerobic conditions is substantial well below 1000 ppm and still detectable in the 1−10 ppm region—just above the H_2 concentration in the lower atmosphere. This demonstrates the extreme selectivity of this hydrogenase and its ability to sequester H_2 in air.

This study establishes for the first time the link between the in vivo threshold levels for H_2 uptake by aerobic H_2-oxidizers (ca. 2 ppm[9,12]) and the behavior of the purified enzymes. Our results show that in electrochemical in vitro experiments, a purified O_2-tolerant hydrogenase can display substantial H_2 oxidation activity even below 10 ppm H_2 in air (against 210 000 ppm O_2). The enzyme thus equips the organism for gaining energy from trace H_2 during aerobic respiration and may account for the fact that *Ralstonia spp.* and other Knallgas bacteria can be isolated from almost all soil and water samples in contact with O_2.[13]

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