Electrocatalytic hydrogen oxidation by an enzyme at high carbon monoxide or oxygen levels

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Use of hydrogen in fuel cells requires catalysts that are tolerant to oxygen and are able to function in the presence of poisons such as carbon monoxide. Hydrogen-cycling catalysts are widespread in the bacterial world in the form of hydrogenases, enzymes with unusual active sites composed of iron, or nickel and iron, that are buried within the protein. We have established that the membrane-bound hydrogenase from the β-proteobacterium *Ralstonia eutropha* H16, when adsorbed at a graphite electrode, exhibits rapid electrocatalytic oxidation of hydrogen that is completely unaffected by carbon monoxide [at 0.9 bar (1 bar = 100 kPa), a 9-fold excess] and is inhibited only partially by oxygen. The practical significance of this discovery is illustrated with a simple fuel cell device, thus demonstrating the feasibility of future hydrogen-cycle technologies based on biological or biologically inspired electrocatalysts having high selectivity for hydrogen.

biohydrogen | electron transfer | energy | fuel cell | hydrogenase

Hydrogenases prevail throughout the microbial world and are essential to hydrogen metabolism (1). X-ray crystallography in conjunction with Fourier transform infrared spectroscopy has revealed that the active site structure of the [NiFe] hydrogenases from *Desulfovibrio gigas* (*Dg*) (2) and *D. vulgaris* Miyazaki F (3, 4) is a bimetallic site having Ni and Fe centers linked by bridging cysteinyl S, with the Fe additionally coordinated by one CO and two CN^- ligands. Crystallographic experiments on the enzyme from *D. fructosovorans* involving infusion of Xe have revealed the likely positions of "gas channels" for transport of small molecules such as H_2, O_2, and CO to and from the active site (5).

The vast majority of hydrogenase-containing microorganisms, including the *Desulfovibrio* species, live under anaerobic or semianaerobic conditions, and like their hosts, the hydrogenases are usually highly sensitive to O_2 (1). However, some bacteria are able to gain energy from H_2 oxidation under aerobic conditions. A well studied example is *Ralstonia eutropha* (*Re*, formerly *Alcaligenes eutrophus*) strain H16 a β-proteobacterium that hosts three physiologically distinct [NiFe] hydrogenases (6–8). One of these enzymes, the membrane-bound hydrogenase (MBH), is coupled via a *b*-type cytochrome to the respiratory chain. Sequence similarity shows that this enzyme belongs to the family of [NiFe] hydrogenases having a large subunit containing the Ni—Fe catalytic center and a small electron-transferring subunit accommodating three iron-sulfur clusters (9). The MBH enables *Re* to grow on H_2 as the sole energy source even under ambient levels of O_2. The exceptional tolerance of *Re* MBH to O_2 (10) inspired us to assess its electrocatalytic activity under extremely demanding conditions, including the presence of CO, which is the classic inhibitor of hydrogen-cycling catalysts (11). This inhibition is rationalized on the basis that activation of H_2 by transition metals requires it to form a bond that involves back donation of d-orbital electron density from the metal into the antibonding σ orbital of H_2 (12, 13). A similar electronic principle is well known to be responsible for the strong binding of π-acceptor ligands such as CO and O_2.

Methods

Preparation of *Re* MBH. *Re* MBH was overproduced by using a broad host-range plasmid harboring all genes necessary for MBH synthesis, maturation, and transcriptional regulation (14). A *Strep*-tag II sequence was fused to the 3′ end of the MBH small subunit gene, *hoxK*, to facilitate purification. Details of the construction will be described elsewhere. *Re* cells containing the MBH overproduction plasmid were grown at 30°C in fructose-glycerol mineral medium in the presence of 80% H_2/10% CO_2/10% O_2. After 48 h of continuous shaking the cells were collected by centrifugation, resuspended in buffer A (50 mM Tris·HCl, pH 8.0/50 mM NaCl) and broken by passage three times through a French pressure cell. The membranes were separated by ultra-centrifugation (1 h at 90,000 × *g* and 4°C). and the MBH was solubilized by incubating the membranes at 4°C in 7.5 vol/g buffer A containing 2% Triton X-114. The cleared solubilizate was applied to a *Strep*-Tactin Superflow column, which was then washed with 8-column volumes of buffer A by using a BioCAD Sprint purification system. The *Strep*-tagged MBH was eluted with 6-column volumes of buffer A containing 5 mM desthiobiotin. Fractions containing MBH were combined and concentrated.

Purification of *Trametes versicolor* (*Tv*) Laccase. Crude powdered extract of *Tv* laccase (Fluka) was suspended in sodium acetate buffer (50 mM, pH 5.5). The same buffer was used throughout the purification. Insoluble material was removed by centrifugation, and the extract was applied to a DEAE Toyopearl 650M column, washed with buffer, and released from the resin with buffer containing ammonium sulfate (100 mM). Laccase-containing fractions were diluted 10-fold with buffer and applied to a Q-Sepharose column (Amersham Pharmacia Biosciences), washed with buffer, and eluted with a 0–100 mM ammonium sulfate gradient in buffer.

Protein Film Voltammetry. Before adsorption of each enzyme film, the rotating pyrolytic graphite "edge" electrode (0.03 cm^2) (15), was polished with a slurry of 1-μm alumina, sonicated, and rinsed in deionized water. A typical film of *Re* MBH was prepared by pipetting dilute enzyme solution (1.5 μl at 0.1 mg/ml) onto the electrode surface and then withdrawing the solution with a pipette. The procedures for electrochemical experiments and preparation of films of *Allochromatium vinosum* (*Av*) MBH have been described in ref. 16, and films of *Dg* hydrogenase were formed in an identical manner.

Experiments were performed in aqueous mixed buffer so-

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Abbreviations: *Av*, *Allochromatium vinosum*; *Dg*, *Desulfovibrio gigas*; MBH, membrane-bound hydrogenase; *Re*, *Ralstonia eutropha*; *Tv*, *Trametes versicolor*.

See Commentary on page 16911.

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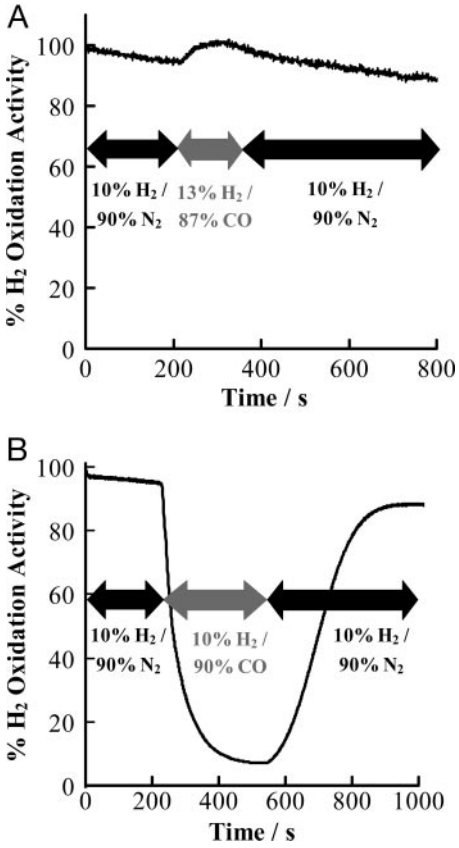

Fig. 1. Oxidation of H_2 in the presence of CO. H_2 oxidation activity measured as an electrocatalytic current at a pyrolytic graphite electrode coated with the membrane-bound hydrogenase from *Re* (*A*) and *Av* (*B*). In both cases, the gas composition in the headspace of the electrochemical cell was varied as indicated, and the electrode was rotated at 2,000 rpm (EG&G model 636 rotator) in a mixed aqueous buffer solution containing NaCl (100 mM, pH 5.6) as an electrolyte at 30°C. The electrode potential was polarized at +142 mV (*A*) and +100 mV (*B*).

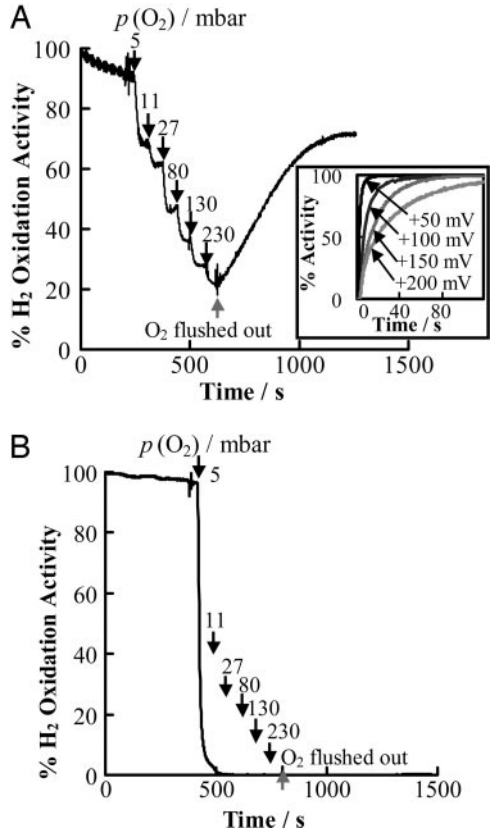

Fig. 2. The effect of O_2 partial pressure (pO_2) on the H_2 oxidation activity of electrode-adsorbed films of the membrane-bound hydrogenase from *Re* (*A*) and *Av* (*B*). In each case, the electrode was polarized at +142 mV and the electrochemical cell was initially flushed with H_2. At 240 s (*A*) and 400 s (*B*), the cell was sealed and pO_2 (mbar) was increased as indicated; at 650 s (*A*) and 815 s (*B*), the cell gas valves were opened and O_2 was flushed out with H_2. *A Inset* shows the rate of recovery of activity for a film of the *Re* enzyme after potential steps to the values indicated, following introduction and subsequent removal of O_2 (200 μM) at +392 mV.

lution containing NaCl (100 mM) at pH 5.6, 30°C, with the electrode rotated at 2,000 rpm (EG&G model 636 rotator) in a sealed glass electrochemical cell at atmospheric pressure. Importantly, no enzyme is present in solution, removing the possibility of exchange with molecules that have not been subject to potential control. The current recorded when H_2 is introduced into the electrochemical cell and the electrode is polarized at +100 to +150 mV reports directly on the H_2 oxidation activity of the adsorbed enzyme sample. Hydrogen, Protec10 (10% H_2 in N_2), and O_2 were purchased from Air Products; CO (research grade, 100.000% purity) and N_2 (oxygen-free) were purchased from BOC. Specific mixtures of H_2 in CO were prepared by combining the gases in a stainless steel pressure vessel. All gases were used without further purification.

Fuel Cell Measurements. Enzyme films were prepared by soaking pyrolytic graphite strips (edge area 0.7 cm^2, freshly polished with 1-μm alumina for the hydrogenases, or with coarse sandpaper for the laccase) in dilute enzyme solution for 5 min (*Re* MBH) or 20 min (*Av* MBH or *Tv* laccase). Hydrogenase films were prepared in a glove box and then stored in anaerobic buffer solution during removal from the box, whereas laccase films were prepared in air. Electrodes were positioned in a beaker containing aqueous citrate buffer (100 mM, pH 5) with H_2 and air inlet tubes positioned close to the hydrogenase and laccase electrodes, respectively. Variable loads (R = 10 Ω to 68 MΩ) were applied by connecting a resistor between the electrodes, and voltages (V) were measured via a voltmeter (Keithley 195A Digital Voltmeter) connected across the cell. Power (P) was calculated from values of V at each R according to $P = V^2/R$.

Results and Discussion

Hydrogenases are noted for their strong inhibition by CO, but unlike Pt-based catalysts, activity is restored when CO is removed (17). In contrast, as shown in Fig. 1*A*, the activity of *Re* MBH is completely unaffected by CO. The electrode is polarized at +142 mV, and the activity of the enzyme film is measured directly from the current resulting from H_2 oxidation (18). The electrochemical cell solution is first equilibrated with 10% H_2:90% N_2 (the slow decrease in current with time is attributed to dissociation of enzyme molecules from the electrode that is rotated to overcome limitations of substrate transport to the enzyme). At 220 s, the gas mixture is changed to 13% H_2:87% CO; remarkably this gas exchange results in an increase in current because of the slightly higher H_2 composition of the mixture. Taking into account the background drop in current due to the gradual loss of enzyme from the electrode, the current is restored to the original level when the gas mixture is returned to 10% H_2:90% N_2 at 300 s. Switching between 10% H_2:90% N_2 and 10% H_2:90% CO results in no detectable changes in the

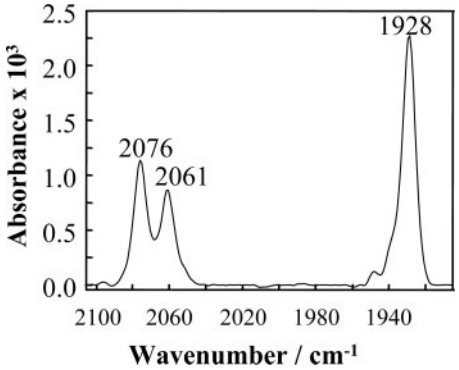

Fig. 3. Fourier transform infrared absorbance spectrum for as-isolated membrane-bound hydrogenase from *Re* (25 mg ml^{-1}). The spectrum was recorded on a Bruker IFS66V/S spectrometer equipped with a liquid nitrogen-cooled MCT detector at a spectral resolution of 2 cm^{-1}. The sample compartment was purged with nitrogen, and the sample was held in a temperature-controlled (23°C) gas-tight liquid cell (path length = 50 μm) with CaF_2 windows. Spectra were baseline corrected by using a spline function implemented within OPUS 4.2 software supplied by Bruker.

current (hence, these data are not shown). The enzyme is thus fully active in the presence of a 9-fold excess of CO over H_2.

Furthermore, cyclic voltammetry (data not shown) in which the electrode potential is scanned across a range of values confirms that introduction of CO at any potential (−558 to +242 mV) has no effect on the hydrogen oxidation activity of *Re* MBH. For comparison, the gas exchange experiment was performed with films of [NiFe] hydrogenases from two other organisms, *Av* (Fig. 1*B*) and *Dg*. For both of these enzymes, hydrogen oxidation activity is lost immediately and completely on introduction of CO but is regained on removal of the inhibitor.

We now discuss the effect of O_2 on the activity of *Re* MBH. In the experiments shown in Fig. 2, the cell solution was first equilibrated with H_2 (1 bar) with the electrode held at +142 mV. Stepwise increases in the partial pressure of O_2 (pO_2) lead to diminishing hydrogenase activity (Fig. 2*A*), but a significant current due to H_2 oxidation is clearly observed even at levels of O_2 above that present in air. Activity is recovered quickly and completely as O_2 is flushed out of the solution (taking into account the gradual loss of enzyme from the electrode). The rate of recovery after removal of O_2 increases as the electrode potential is dropped to lower values, reaching a limiting rate of $>0.2\ s^{-1}$ below *ca.* +50 mV at 30°C (see Fig. 2*A Inset*). The rate of loss of activity upon addition of O_2 (data not shown) also increases markedly as the potential is lowered. In stark contrast, in an analogous experiment (Fig. 2*B*), the activity of *Av* MBH is completely abolished by the first addition of O_2 (pO_2 = 5 mbar) and no recovery in activity is recorded during 500 s after removal of O_2. The *Av* MBH shows a negligible rate of recovery at potentials above 0 mV and reaches a limiting rate when the potential is taken below −100 mV. Even at 45°C, this limiting rate is just $0.0025\ s^{-1}$ (19).

We now consider the biological, chemical, and structural implications of the reactions of hydrogenases with CO and O_2. The O_2 tolerance of the *Re* MBH monitored *in vitro* is fully compatible with its biological role: oxidation of H_2 in the presence of oxygen and ability to recover rapidly after exposure to cellular O_2 bursts. Clearly *Re*, as an aerobic organism, has adapted such that its MBH recovers from exposure to O_2 far more quickly and at much milder redox potentials than the *Av* or *Dg* enzymes. For *Re* MBH, the fact that reaction with O_2 and removal of the inhibitory product (reactivation) are both so potential dependent suggests that inhibition is not simply due to O_2 blocking, or being prevented from using, the gas channel. It

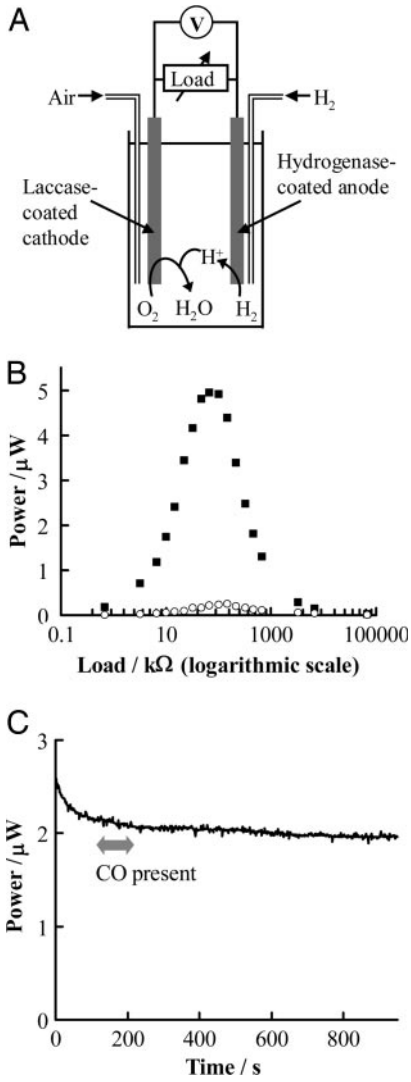

Fig. 4. A membraneless hydrogen-oxygen fuel cell with enzymes as electrocatalysts. (*A*) Diagram showing a simple H_2/O_2 membraneless fuel cell set up in a beaker with pyrolytic graphite edge strips coated with enzymes as specific catalysts. The cathode is coated with laccase from the white rot fungus, *Tv*, and the anode is coated with *Re* membrane-bound hydrogenase (MBH). In *B*, the filled squares (■) show a power output vs. applied load curve for a simple fuel cell set up according to the diagram in *A*. The enzyme-coated surface area of each electrode is ≈0.7 cm^2. Open circles (○) show the power output recorded when the electrode coated with *Re* MBH is replaced by an electrode coated with *Av* MBH. The power output vs. time curve in *C* was recorded when a constant load of 330 kΩ was applied to the fuel cell shown in *A*. During the period marked by a horizontal gray arrow, CO was flushed into the cell solution close to the *Re* MBH-coated electrode.

is also highly unlikely that O_2 will damage buried FeS clusters in a rapid, fully reversible manner. We therefore conclude that O_2 reacts directly at the Ni-Fe active site. Hard sphere diameters for O_2 and CO are similar (3.55 and 3.70 Å, respectively, *cf.* 2.71 Å for H_2, calculated from gas viscosities) (20); consequently, CO must also have access, and the fact that it does not inhibit suggests an inability to form a metal-CO bond. According to structural and spectroscopic studies carried out on the standard [NiFe] hydrogenase from *D. vulgaris* (Miyazaki F), the exogenous (inhibitory) CO molecule binds to the Ni atom (21). It is thus important to know how typical is the active site of *Re* MBH. The infrared spectrum of as-isolated (oxidized) *Re* MBH shows an intense band at 1,928 cm^{-1}, consistent with one endogenous

CO ligand, and bands at 2,076 and 2,061 cm^{-1}, consistent with two endogenous CN^- ligands (Fig. 3). This band pattern is the same as found for standard [NiFe] hydrogenases (22, 23) and suggests strongly that *Re* MBH contains the same complement of permanent CO and CN^- ligands. To see whether CO binding could be detected by infrared, a sample was reduced with dithionite and the spectrum was measured under 1 bar N_2 or 1 bar CO. Although the spectra showed that the sample is only partially reduced by dithionite, the more complex band pattern did not differ depending on whether CO was present (data not shown). The oxidized spectrum was also unchanged upon exposure to CO. Thus, the lack of CO binding is not due to competition by H_2. Although the overall amino acid composition of hydrogenase large subunits is fairly variable, the residues neighboring the Ni-Fe active site are highly conserved (1). The unusual O_2 and CO tolerance of *Re* MBH must therefore be due to the effect of more distant residues that alter the structure of the active site pocket.

Demonstration of the tolerance of *Re* MBH to CO and O_2 has important technological implications. An electrode coated with a film of the *Re* MBH possessing a binuclear Ni-Fe active site represents a previously undescribed H_2 oxidation electrocatalyst that is completely tolerant to CO; thus, even Synthesis Gas (an industrial H_2/CO mixture) would provide a viable H_2 fuel source. Intriguingly, modifications to platinum surfaces that confer increased resistance to CO poisoning often depend on introduction of a second metal, as in the intermetallic materials PtBi, PtIn, or PtPb (24) or the binary alloys PtRu or PtMo (25).

The observation that an electrode coated with *Re* MBH catalyzes H_2 electrooxidation, even in the presence of ambient levels of O_2, suggests that it may be possible to operate a H_2/O_2 fuel cell with no membrane separating the anode and cathode, i.e., in a single compartment. As a demonstration of this concept, we conducted a very simple experiment in which two strips of pyrolytic graphite, coated respectively with *Re* MBH and laccase from the white rot, *Tv*, were dipped in a beaker containing aqueous buffer flushed with H_2 and air as shown in Fig. 4*A*. Electrode-adsorbed *Tv* laccase catalyzes the reduction of O_2 to water at high potential (>800 mV) under these conditions. The system reaches an open circuit voltage of ≈970 mV. With a variable load (*R*) connected between the electrodes, the variation in voltage (*V*) measured across this simple fuel cell gives rise to the power-against-load plot shown in Fig. 4*B* (solid squares), showing a maximum power output of ≈5 μW. As a control, the experiment was repeated with electrodes coated with *Av* MBH and *Tv* laccase in the beaker. In this case, the open circuit voltage is only 480 mV, dropping rapidly as the applied load is lowered, to give a maximum power of only 0.2 μW as shown in Fig. 4*B* (open circles). Fig. 4*C* shows a plot of power output against time recorded as a constant load of 330 kΩ is applied to the fuel cell with electrodes coated with *Re* MBH and *Tv* laccase. Before time 0, the cell was held under open circuit conditions. An initial rapid drop in power is observed over the first 100 s, after which the output is reasonably stable over >15 min. During the period indicated by the horizontal gray arrow, CO was flushed into the beaker close to the *Re* MBH electrode. No detectable change in current was observed, confirming that *Re* MBH will catalyze oxidation of H_2, even from CO-contaminated fuels. A hydrogenase enzyme with the properties of *Re* MBH, or a synthetic catalyst inspired by this discovery, thus opens the way for significant breakthroughs in fuel cell technologies.

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