Supporting information

Enhanced Oxygen-Tolerance of the Full Heterotrimeric Membrane-Bound [NiFe]-Hydrogenase of *Ralstonia eutropha*

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Experimental Section

Media and growth conditions for *R. eutropha* H16 have been described elsewhere.[1] Cytoplasmic membranes were prepared from total membranes of *R. eutropha* HF632 (MBH expression strain) and HF690 (Δ*hoxK* control strain lacking the MBH small subunit) [2] by sucrose gradient centrifugation, as previously described[3].

Template stripped gold (TSG) surfaces, self-assembled monolayers (SAMs), and tethered Bilayer Lipid Membranes (tBLMs) were prepared as previously described.[3,4] Briefly, *E. coli* polar lipids were mixed with ubiquinone-10 (1% w/w) or menaquinone-7 (2% w/w), as indicated in the communication, and vesicles were prepared by extrusion through a track-etched membrane (200 nm). These liposomes were mixed with cytoplasmic membrane extracts to obtain a ratio of dry *E. coli* polar lipids to dry total protein (from the cytoplasmic membrane) of 10:1 (w/w; Figure 2b) and 10:4 (w/w; Figure 2a,c and d and Figure 3). The obtained mixture was subjected to three freeze-thaw cycles and was extruded again through a track-etched membrane (200 nm) to obtain mixed vesicles. We have previously noted that commercially obtained *E. coli* polar lipids contain trace amounts of ubiquinone-8 (footnote 56 in reference [5]). It is likely that also the cytoplasmic membrane extracts from *R. eutropha* contain ubiquinone-8. Accordingly, the mixed vesicle preparation with 2% (w/w) menaquinone-7 (without added ubiquinone-10) still contains trace amounts of ubiquinone-8.

SAMs were obtained by incubating TSG surfaces in isopropanol containing different ratios of EO3-cholesteryl to 6-mercaptohexan-1-ol to give a total of 1 mM thiol compounds (~16 h).[4] TBLMs were formed by adding mixed vesicles, at a final lipid concentration of 0.45 mg/mL, to a mixed SAM (with a surface coverage of EO3-cholesteryl between 30-50%) in the presence of 10 mM $CaCl_2$. The formation of the bilayer was verified by monitoring the drop of the double layer capacitance using electrochemical impedance spectroscopy (EIS) measurements as previously described.[3,4] After the formation of the mixed tBLM (~1h), the surface was rinsed with MOPS buffer (20 mM MOPS, 30 mM Na_2SO_4, pH 7.4) to remove remaining vesicles and with 1mM EDTA-MOPS buffer to remove Ca^{2+} ions. The integrity of the bilayer-SAM system was checked after washing and throughout the experiment via EIS. The EIS data confirmed that the tBLM, including the S-Au bond in the underlying SAM, was stable in the potential range of -0.4 V to +0.6 V at high temperature (up to 50 °C) under nitrogen, hydrogen and/or oxygen conditions. Based on the area of the redox signals, ubiquinone coverage was typically determined to lie between 6-8 pmol/cm^2. For the pH screening experiments a mixed buffer was used containing MOPS (3-morpholinopropane-1-sulfonic acid), MES (2-(*N*-morpholino)ethanesulfonic acid), TAPS (3-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]propane-1-sulfonic acid), CHES (2-(Cyclohexylamino)ethanesulfonic acid), and sodium citrate at 20 mM concentration, and 30 mM Na_2SO_4. Other experiments were performed in 20 mM MOPS, 30 mM Na_2SO_4, pH 7.4.

Electrochemical measurements were carried out in a three electrode configuration electrochemical cell described previously, using either a silver-silver chloride or mercury-mercury sulfate reference electrode.[3] All potentials are quoted versus the SHE ($E_{SHE}=E_{Hg/Hg2SO4}$ + 651 mV at 25 ^oC; $E_{SHE}=E_{Ag/AgCl}$ + 199 mV at 25 ^oC). Unless stated otherwise, the electrochemical cell was housed in a Faraday cage (for electrical noise minimization) inside a nitrogen filled glove box (MBraun Lab Master sp) which maintains oxygen levels below 0.1 ppm. Gases were bubbled into the cell solution at constant flow rates using two gas mass flow controllers (Smart-Trak Series 100, Sierra Instruments, accuracy: 1% of full range). The gases used were hydrogen (BOC), nitrogen (oxygen-free, BOC), air (BOC) and a 95% nitrogen-5% hydrogen mixture (BOC). Electrochemical measurements were carried out using an Autolab (Eco-chemie) electrochemical analyzer equipped with a PGSTAT30 potentiostat, SCANGEN module and a FRA2 frequency analyzer.

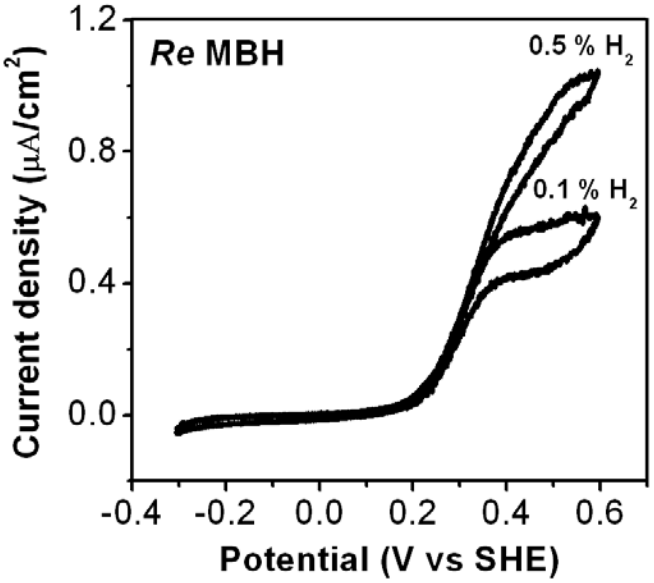

Figure S1. CVs recorded under 0.1 % (0.8 μM H_2 in solution) and 0.5 % H_2 (4 μM H_2 in solution) in N_2 at 1 mV/s, pH 7.4, 30 ^oC. Some hysteresis is observed in the high potential region. This could be due to diffusion limitation of hydrogen, but it cannot be excluded this is due to some reversible enzyme inactivation. However, the extent is much smaller than reported for the hydrophilic heterodimeric MBH under the same conditions.

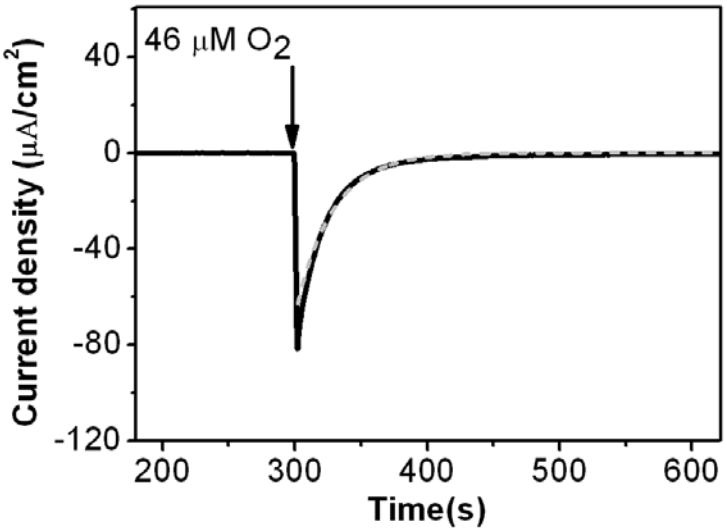

Figure S2. Chronoamperogram from a bare Au electrode measured at -0.353 V. An air saturated aliquot of buffer was inserted into the cell solution after 300 s from the starting point leading to a final O_2 concentration of ~ 46 μM. The cell was continuously purged with H_2 (30 ^oC; pH 7.4). The value of the time constant for exponential gas removal (τ) was determined by analyzing the decay of the reduction current following O_2 injection using the following equation: $C(t)=C(0)\cdot\exp(-t/\tau)$ (*C* is concentration, *t* is time). The dashed trace (light gray) is the exponential fit to the current decay.

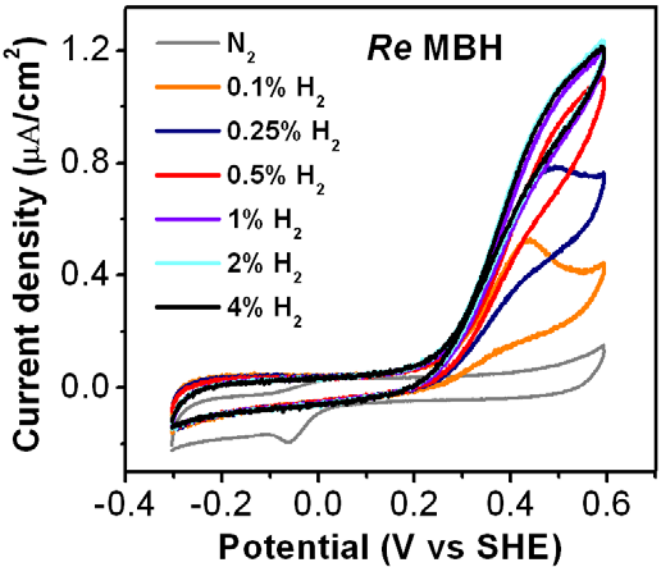

Figure S3. CVs recorded at increasing H_2 concentrations (10 mV/s; 30 °C; pH 7.4). An average value of 2.5±0.5 µM was determined for $K_{M(app)}$. The current values used for calculating the $K_{M(app)}$ were taken from the return scan at potentials of 0.397 and 0.497 V. The activity of ubiquinol oxidases can be observed at potentials below -0.1 V in the scan recorded under N_2 with trace amounts of O_2.

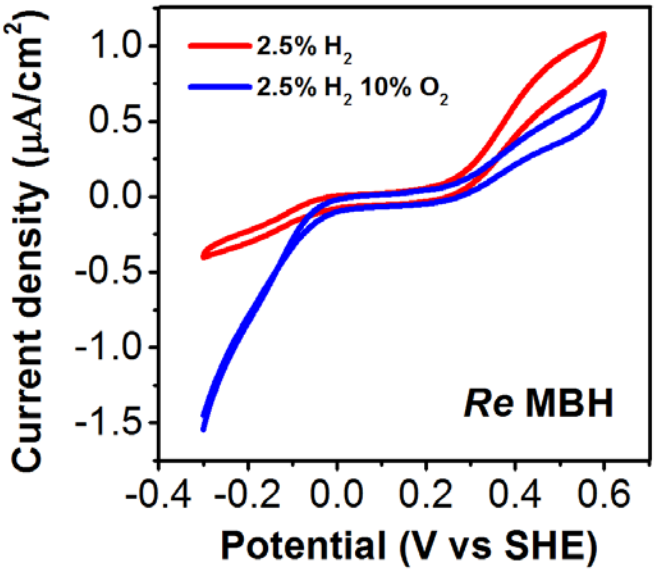

Figure S4. CVs recorded at 2.5% H_2 without and with 10% O_2 (5 mV/s; 30 °C; pH 7.4). Gas mixtures were prepared by making 1:1 mixtures of 5% hydrogen in nitrogen and either nitrogen or air, respectively. Both scans were recorded on the bench (i.e., outside the anaerobic glovebox) and the voltammogram in the absence of O_2 (red trace) indicates the presence of trace amounts of O_2. The latter results in the catalytic wave at potentials < -0.1 V, at which potential the ubiquinone pool becomes reduced and is reoxidised by the membrane-enzyme, ubiquinol oxidase, which is also present in the membrane extracts of *R. eutropha* HF632 and catalytically reduces oxygen. The tail at < -0.1 V in the presence of 10% O_2 is believed to be a combination of ubiquinol oxidase activity and some direct reduction of oxygen by the gold electrode. The reduction in current at potentials > 0.3 V is either due to a reduced activity of MBH in the presence of oxygen or due to the fact that ubiquinol oxidase reoxidises part of the quinone pool instead of the electrode.

References

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