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

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Supporting Information

ABSTRACT: Hydrogenases are oxygen-sensitive enzymes that catalyze the conversion between protons and hydrogen. Water-soluble subcomplexes of membrane-bound [NiFe]-hydrogenases (MBH) have been extensively studied for applications in hydrogen–oxygen fuel cells as they are relatively tolerant to oxygen, although even these catalysts are still inactivated in oxidative conditions. Here, the full heterotrimeric MBH of *Ralstonia eutropha*, including the membrane-integral cytochrome *b* subunit, was investigated electrochemically using electrodes modified with planar tethered bilayer lipid membranes (tBLM). Cyclic voltammetry and chronoamperometry experiments show that MBH, in equilibrium with the quinone pool in the tBLM, does not anaerobically inactivate under oxidative redox conditions. In aerobic environments, the MBH is reversibly inactivated by O₂, but reactivation was found to be fast even under oxidative redox conditions. This enhanced resistance to inactivation is ascribed to the oligomeric state of MBH in the lipid membrane.

Hydrogenases are complex microbial metalloenzymes which catalyze the reversible oxidation of H₂ to protons at rates comparable to those normally achieved by Pt. They are widespread in the microbial world where they are used to dispose of excess reducing power (H₂ production) or to produce energy (H₂ oxidation). Hydrogenases have thus been intensively studied both as an inspiration to design inorganic catalysts and as biocatalysts themselves. Based on the metal content of the active site, H⁻⁻H₂ interconverting hydrogenases have been classified into [NiFe]- and [FeFe]-hydrogenases. [FeFe]-hydrogenases have high turnover frequencies for H₂ production, but they are inactivated by trace amounts of O₂. [NiFe]-hydrogenases, by contrast, are generally less sensitive to O₂ inactivation and biased toward H₂ oxidation.

[NiFe]-hydrogenases have been further subdivided into “standard” (O₂-sensitive) and O₂-tolerant hydrogenases, and many studies have focused on the elucidation of the origins of the O₂ tolerance. The membrane-bound hydrogenase (MBH) from the β-proteobacterium *Ralstonia eutropha* is one of the best studied O₂-tolerant [NiFe]-hydrogenases. It is an uptake hydrogenase that links H₂ oxidation to quinone reduction and has an outstanding O₂ tolerance, capable of maintaining a high level of activity in the presence of air supplemented with low H₂ concentrations. As many other uptake [NiFe]-hydrogenases, it consists of three subunits, one of which is an integral membrane protein (Figure 1). The [NiFe] active center is located in the large subunit (α), and three [FeS] clusters are aligned in the small subunit (β) forming an electron relay. The third subunit is a diheme cytochrome *b*₅₆₂, which anchors the protein complex to the cytoplasmic membrane and transfers electrons from H₂ oxidation to the respiratory chain via the quinone pool. Both ubiquinone and menaquinone have been proposed to be the native substrate.

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The O₂ tolerance of the MBH and of closely related enzymes has been attributed to the “special” design of the unusual proximal [4Fe-3S] cluster, which has the ability to provide two electrons in a narrow potential range, helping to keep a reducing environment when the MBH reacts with oxygen. 11 When O₂ reacts at the active site, this cluster ensures the formation of a state upon reacting with O₂, which usually prevents us to reach potentials at which MBH is fully active as the tBLM system is damaged by potentials higher than 0.6 V. The shift in potential is a consequence of the pH dependence of the ubiquinol oxidation potential. We also propose that the absence of clear current plateaus at high potential (Figure 2) is due to the particular kinetic properties of the electrochemical oxidation of ubiquinol, which is coupled or “gated” by slow proton release in the lipid bilayer. 12 Above pH 8 the enzymatic activity sharply drops, and no recovery is observed when the pH is subsequently lowered, suggesting denaturation of MBH at high pH. The MBH in the tBLM is stable at temperatures up to 50 °C, although some loss of activity is observed on time-scales in the order of hours at temperatures above 30 °C (Figure 2b). Consequently all the following experiments were performed at 30 °C and pH 7.4.

Control experiments were carried out by recording cyclic voltammograms (CVs) of tBLMs prepared from cytoplasmic membranes lacking the αβ subcomplex of the MBH, which showed no H₂ oxidation activity.

CVs recorded under 100% N₂ (Figure 2, gray lines) show no catalytic oxidation waves, instead uncovering the oxidation and reduction signals of the ubiquinone pool in the tBLM. The large peak separation of the ubiquinone electrochemistry has previously been studied in detail and is caused by the coupling of the electron transfer with protonation/deprotonation steps, which are slow due to the lipid bilayer environment. 13 The onset of H₂ oxidation (black line) coincides with ubiquinol oxidation (Figure 2c), confirming the fact that the electron transfer between the MBH and the electrode takes place via the quinone pool. A clear feature in all the catalytic oxidation waves in Figure 2 is the absence of any anaerobic inactivation at high potential. Previous studies have shown that the anaerobic inactivation of the heterodimeric αβ subcomplex of MBH is more pronounced at low substrate concentrations and slow scan rates. 14 Therefore, CVs were recorded at 1 mV/s under 0.5% (4.0 μM) and 0.1% (0.8 μM) H₂ (insert in Figure 2c and Figure S1). In either condition, no decrease in current is observed as the potential is swept toward positive values, which confirms that the heterotrimERIC MBH in equilibrium with the quinone pool displays little or no anaerobic inactivation even in substrate limiting conditions.

The presence of a hydrophilic subcomplex of MBH is immobilized at an electrode interface using a so-called tethered bilayer lipid membrane (tBLM) (Figure 1). 16 Cytoplasmic membrane extracts from R. eutropha, containing MBH, are tethered to an electrode surface using cholesterol-based anchor molecules. The full heterotrimERIC structure is retained as the MBH remains in a native-like membrane environment. By incorporating ubiquinone in the tBLM, the native catalytic function of the MBH, namely H₂-ubiquinone oxidoreduction, can be studied, where the redox state of the quinone pool is controlled by the potential applied to the electrode (Figure 1). We show that the full heterotrimERIC MBH in a lipid environment does not display anaerobic (oxidative) inactivation, as the hydrophilic subcomplexes do, and that O₂-inactivated MBH rapidly reactivates under oxidative conditions even when the quinone pool is fully oxidized.

Experiments examining the influence of H₂ and temperature on enzyme activity were carried out to determine the optimum conditions for monitoring the catalytic activity (Figure 2). The optimum pH value for H₂ oxidation activity seems to lie in the range of 7 to 8, unlike the heterodimeric αβ subcomplex for which an optimum between 4.5 and 6.5 was determined (Figure 2a). 5,6a,c,f,10,12 A similar difference in pH optimum was reported in a study employing spectrophotometric assays. 14 However, we note that at lower pH, the oxidation wave shifts to higher potentials, preventing us to reach potentials at which MBH is fully active as the tBLM system is damaged by potentials higher than 0.6 V. The shift in potential is a consequence of the pH dependence of the ubiquinol oxidation potential. We also propose that the absence of clear current plateaus at high potential (Figure 2) is due to the particular kinetic properties of the electrochemical oxidation of ubiquinol, which is coupled or “gated” by slow proton release in the lipid bilayer. 12 Above pH 8 the enzymatic activity sharply drops, and no recovery is observed when the pH is subsequently lowered, suggesting denaturation of MBH at high pH. The MBH in the tBLM is stable at temperatures up to 50 °C, although some loss of activity is observed on time-scales in the order of hours at temperatures above 30 °C (Figure 2b). Consequently all the following experiments were performed at 30 °C and pH 7.4.

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To confirm that also menaquinone can act as electron acceptor of MBH, as previously proposed, experiments were performed with added menaquinone (Figure 2d, see SI for details). Menaquinone is oxidized at a lower potential than ubiquinone, and consequently the onset of hydrogen oxidation is visible from about −0.1 V onward, about 0.3 V lower. The reduction potential of menaquinone is only −0.2 V lower than ubiquinone. However, as already mentioned, the quinone oxidation in tBLMs is coupled to the deprotonation of the quinol.18 Apparently, differences between deprotonation rates and/or pKₐ shifts between menaquinone and ubiquinone cause an additional 0.1 V shift.

In Figure 2d, a slight shoulder around 0.3 V is visible, which is due to trace amounts of ubiquinone-8 present in the tBLM. This ubiquinone originates from the Escherichia coli lipid extract as well as the cytoplasmic membrane extracts from R. eutropha that were used to prepare the TBLM (see SI for details). Similar to the experiments with ubiquinone, no inactivation is observed at high potentials.

Using a method developed by Léger et al., the apparent Michaelis−Menten constant for hydrogen (K_M(app)) was calculated with ubiquinone as electron acceptor. The method involves the addition of a H₂-saturated aliquot to a stirred working solution which is continuously flushed with N₂, while the working electrode potential is maintained at a fixed positive value (Figure 3a). The value of K_M(app) can be calculated by:

\[
K_{M(app)} = \frac{M_{(app)}}{C_{(t)}} = \frac{M_{(app)}}{C(0) \exp(-t/\tau)}
\]

where C(t) is the concentration of hydrogen at time t, and τ is the time constant for exponential gas removal and was determined to be 22 s under these conditions (see Figure S2).

![Figure 3. (a) Chronoamperogram showing the evolution of the MBH activity after the injection of a H₂-saturated aliquot of buffer into the cell solution flushed with N₂ (+0.497 V vs SHE, 30 °C, pH 7.4). (b) Chronoamperometry of the MBH (+0.397 V vs SHE, 100% H₂, pH 7.4, 30 °C). The current is used to determine the hydrogen oxidation activity, which is normalized to 100% at the start of the experiment. An aliquot (one-fourth volume of the final cell volume) of air-saturated buffer was inserted into the electrochemical cell at 270 s. The exponential decay of the O₂ concentration was plotted according to the equation: C(t) = C(0) exp(−t/τ), C is concentration, t is time, and τ is the time constant for exponential gas removal and was determined to be 22 s under these conditions (see Figure S2).](image)

Analyzing the sigmoidal current decay as the gases (in this case H₂) are removed from solution following an exponential decay. The exponential time-dependency of gas removal from solution was confirmed through independent experiments (see Figure S2). Table 1 shows that K_M(app) increases with the applied electrode potential, suggesting that at high H₂ concentrations ubiquinol ubiquinone cycling might be limiting the rate of H₂ oxidation, especially at potentials below 0.5 V. CVs were measured at increasing H₂ concentration to further support the

Table 1. Value of K_M(app) (± SEM) at Different Potentials*<br>
| Potential (V vs SHE) | K_M(app) (μM) |
|----------------------|--------------|
| +0.397               | 1.5 ± 0.3 (n = 3) | 2.1 ± 0.9 (n = 8) | 9.2 ± 2.7 (n = 5) |
| +0.497               | 1.5 ± 0.3 (n = 3) | 2.1 ± 0.9 (n = 8) | 9.2 ± 2.7 (n = 5) |
| +0.597               | 1.5 ± 0.3 (n = 3) | 2.1 ± 0.9 (n = 8) | 9.2 ± 2.7 (n = 5) |

*Number of independent experiments is given by n.
[FeS] clusters is short, supporting the hypothesis that intermolecular electron transfer is possible.20

■ ASSOCIATED CONTENT

2 Supporting Information

Experimental details and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Jones, A. K.; Sillery, E.; Albracht, S. P. J.; Armstrong, F. A. Chem. Commun. 2002, 866.

(2) (a) Vignais, P. M.; Billoud, B. Chem. Rev. 2007, 107, 4206. (b) Fritsch, J.; Lenz, O.; Friedrich, B. Nature Rev. Microbiol. 2013, 11, 106.

(3) Vincent, K. A.; Cracknell, J. A.; Clark, J. R.; Ludwig, M.; Lenz, O.; Friedrich, B.; Armstrong, F. A. Chem. Commun. 2006, 5033.

(4) (a) Ciaccavaca, A.; De Poulpiquet, A.; Teher, V.; Giudici-Orticci, M. T.; Tingry, S.; Innoccenti, C.; Lojou, E. Electrochem. Commun. 2012, 23, 25. (b) De Poulpiquet, A.; Ciaccavaca, A.; Gadiou, R.; Goulon, S.; Giudici-Orticci, M. T.; Mano, N.; Lojou, E. Electrochem. Commun. 2014, 42, 72. (c) Wait, A. F.; Parkin, A.; Morley, G. M.; dos Santos, M.; Armstrong, F. A. J. Phys. Chem. C 2010, 114, 12003. (d) Xu, L.; Armstrong, F. A. Energy Environ. Sci. 2013, 6, 2166. (e) Hamburger, M.; Gervaldo, M.; Svedruzic, D.; King, P. W.; Gust, D.; Ghirardi, M.; Moore, A. L.; Moore, T. M. Am. Chem. Soc. 2008, 130, 2015. (f) Le Goff, A.; Artezio, V.; Jousselme, B.; Tean, P. D.; Guillet, N.; Metayé, R.; Fihri, A.; Palacin, S.; Fontecave, M. Science 2009, 326, 1384. (g) Song, L.; Li, J.-P.; Xie, Z.-J.; Song, H.-B. J. Phys. Chem. C 2013, 9, 15. (h) Lubicz, W.; Ogata, H.; Rüdiger, O.; Reijerse, E. Chem. Rev. 2014, 114, 4081.

(8) (a) Lenz, O.; Ludwig, M.; Schubert, T.; Bürstel, I.; Ganskow, S.; Goris, T.; Schwarze, A.; Friedrich, B. ChemPhysChem 2010, 11, 1107. (b) Frielingsdorf, S.; Schubert, T.; Pohlmann, A.; Lenz, O.; Friedrich, B. Biochemistry 2011, 50, 10836.

(9) (a) Ludwig, M.; Cracknell, J. A.; Vincent, K. A.; Armstrong, F. A.; Lenz, O. J. Biol. Chem. 2009, 284, 465. (b) Cracknell, J. A.; Wait, A. F.; Lenz, O.; Friedrich, B.; Armstrong, F. A. Proc. Natl. Acad. Sci. U.S.A. 2010, 106 (49), 20681.

(10) Fritsch, J.; Scheer, P.; Frielingsdorf, S.; Kroschinsky, S.; Friedrich, B.; Lenz, O.; Spahn, C. M. T. Nature 2011, 479, 249.

(11) Bernhard, M.; Benell, B.; Hochkoeppler, A.; Zannoni, D.; Friedrich, B. Eur. J. Biochem. 1997, 248, 179.

(12) (a) Goris, T.; Wait, A. F.; Saggau, M.; Fritsch, J.; Heidary, N.; Stein, M.; Zebger, I.; Lendzian, F.; Armstrong, F. A.; Friedrich, B.; Lenz, O. Nat. Chem. Biol. 2011, 7, 310. (b) Shomura, Y.; Yoon, K.-S.; Nishihara, H.; Hiyugi, Y. Nature 2011, 479, 253. (c) Pandela, M. E.; Nitschke, W.; Infossi, P.; Giudici-Orticci, M. T.; Bill, E.; Lubicz, W. Proc. Natl. Acad. Sci. U.S.A. 2011, 108, 6097.

(13) (a) Ogata, H.; Hirota, S.; Nakahara, A.; Komori, H.; Shibata, N.; Kano, K.; Hiyugi, Y. Structure 2011, 13, 1635. (b) Jones, A. K.; Lamle, S. E.; Pershad, H. R.; Vincent, K. A.; Albracht, S. P. J.; Armstrong, F. A. J. Am. Chem. Soc. 2003, 125, 8505. (c) Saggau, M.; Zebger, I.; Ludwig, M.; Lenz, O.; Friedrich, B.; Hildebrandt, P.; Lendzian, F. J. Biol. Chem. 2009, 284, 16264. (d) Lamle, S. E.; Albracht, S. P. J.; Armstrong, F. A. J. Am. Chem. Soc. 2004, 126, 14899. (e) Lamle, S. E.; Albracht, S. P. J.; Armstrong, F. A. J. Am. Chem. Soc. 2004, 127, 6595.

(14) (a) McIntosh, C. L.; Germer, F.; Schulz, R.; Appel, J.; Jones, A. K. J. Am. Chem. Soc. 2011, 133, 11308. (b) Leger, C.; Bertrand, B. Chem. Rev. 2008, 108, 2379. (c) Lojou, E.; Giudici-Orticci, M.-T.; Blanco, P. J. Electroanal. Chem. 2005, 577, 79. (d) Armstrong, F. A.; Belsey, N. A.; Cracknell, J. A.; Goldet, G.; Parkin, A.; Reisner, E.; Vincent, K. A.; Wait, A. F. Chem. Soc. Rev. 2009, 38, 36.

(15) (a) Ciaccavaca, A.; Infossi, P.; Ilbert, M.; Guiral, M.; Lecomte, S.; Giudici-Orticci, M.-T.; Lojou, E. Angew. Chem., Int. Ed. 2012, 51, 953. (b) Rudiger, O.; Abad, J. M.; Hatchikian, E. C.; Fernandez, V. M.; De Lacey, A. J. Am. Chem. Soc. 2005, 127, 16008. (c) Gutierrez-Sanz, O.; Marques, M.; Pereira, I. A. C.; De Lacey, A. L.; Lubicz, W.; Rudiger, O. J. Phys. Chem. Lett. 2013, 4, 2794.

(16) (a) Jeuk, L. J. C.; Connell, S. D.; Henderson, P. J. F.; Gennis, R. B.; Evans, S. D.; Bushby, R. J. J. Am. Chem. Soc. 2006, 128, 1711. (b) Weiss, S. A.; Bushby, R. J.; Evans, S. D.; Henderson, P. J. F.; Jeuk, L. J. C. Biochem. J. 2009, 417, 555.

(17) Schink, B.; Schleger, H. G. Biochim. Biophys. Acta 1979, 567, 315.

(18) (a) Gordillo, G. J.; Schiffer, J. D. Faraday Discuss. 2000, 116, 89. (b) Jeuk, L. J. C.; Bushby, R. J.; Evans, S. D. Electrochem. Commun. 2007, 9, 610.

(19) Leger, C.; Dementin, S.; Bertrand, P.; Rousset, M.; Giudici-Orticci, B. J. Am. Chem. Soc. 2004, 126, 12162.

(20) Volbeda, A.; Darnault, C.; Parkin, A.; Sargent, F.; Armstrong, F. A.; Fontecilla-Camps, J. C. Structure 2013, 21, 184.