Oxygen-tolerant H₂ Oxidation by Membrane-bound [NiFe] Hydrogenases of Ralstonia Species

COPING WITH LOW LEVEL H₂ IN AIR

Marcus Ludwig, James A. Cracknell, Kylie A. Vincent, Fraser A. Armstrong, and Oliver Lenz

Received for publication, May 13, 2008, and in revised form, November 6, 2008
Published, JBC Papers in Press, November 6, 2008, DOI 10.1074/jbc.M803676200

Knallgas bacteria such as certain Ralstonia spp. are able to obtain metabolic energy by oxidizing trace levels of H₂ using O₂ as the terminal electron acceptor. The [NiFe] hydrogenases produced by these organisms are unusual in their ability to oxidize H₂ in the presence of O₂, which is a potent inactivator of most hydrogenases through attack at the active site. To probe the origin of this unusual O₂ tolerance, we conducted a study on the membrane-bound hydrogenase from Ralstonia eutropha H16 and that of the closely related organism Ralstonia metallidurans CH34, which was purified using a new heterologous overproduction system. Direct electrochemical methods were used to determine apparent inhibition constants for O₂ inhibition of H₂ oxidation (K_{H₂}^{app}) for each enzyme. These values were at least 2 orders of magnitude higher than those of “standard” [NiFe] hydrogenases. Amino acids close to the active site were exchanged in the membrane-bound hydrogenase of R. eutropha H16 for those from standard hydrogenases to probe the role of individual residues in conferring O₂ sensitivity. Michaelis constants for H₂ (K_{M}^{H₂}) were determined, and for some mutants these were increased more than 20-fold relative to the wild type. Mutations resulting in membrane-bound hydrogenase enzymes with increased K_{H₂}^{app} or decreased K_{O₂}^{app} values were associated with impaired lithoautotrophic growth in the presence of high O₂ concentrations.

Hydrogenases are active catalysts both in H₂ oxidation and proton reduction (1). Three phylogenetically distinct classes of hydrogenases are found in nature as follows: [FeFe], [NiFe], and [Fe] hydrogenases (2, 3). The basic module of a [NiFe] hydrogenase consists of two subunits, a large subunit that contains the Ni-Fe active site, and a small, electron-transferring subunit that accommodates three Fe-S clusters (4–6). The Ni is coordinated to the protein via four thiol groups from cysteine residues, two of which are bridging ligands to the Fe, which additionally coordinates two CN⁻ and one CO (7, 8). The structure of the active site of the “standard” O₂-sensitive [NiFe] hydrogenase from Desulfovibrio gigas is shown in Fig. 1A.

A crucial feature of hydrogenases is their sensitivity to O₂. Although the [NiFe] hydrogenases are generally more robust toward O₂ damage than the [FeFe] enzymes (9, 10), the vast majority of [NiFe] hydrogenases act under anaerobic conditions in vivo, and their activity is normally subject to reversible inactivation by O₂. Dioxogen is a π-acceptor ligand like H₂ and CO and is expected to enter the active site easily. However, it subsequently behaves as an oxidizing agent, leading to inactive “resting” states. EPR studies on a set of standard [NiFe] hydrogenases, such as those isolated from Allochromatium vinosum, D. gigas, Desulfovibrio vulgaris Miyazaki F, and Desulfovibrio fructosovorans, have identified distinct Ni(III) features associated with two inactive states, known as Ni-A (“Unready”) and Ni-B (“Ready”), and these are also associated with subtly distinct ν(CO) and ν(CN) frequencies in IR spectra (11, 12). In conjunction with crystallographic studies on the D. gigas enzyme, for example, it has been established that Ni-B incorporates a hydroxide ligand into the bridging position between Ni and Fe, whereas further electron density in the active site of Ni-A suggests a bridging peroxide ligand or an O atom bonded to cysteiny1 S; both ligands are released through reductive activation under H₂ (13–15). The ready state of the enzyme is also formed under anaerobic oxidative conditions (10–12, 16).

In contrast to standard hydrogenases, a few [NiFe] hydrogenases, especially those synthesized by Knallgas bacteria, catalyze aerobic H₂ oxidation using O₂ as the terminal oxidant (9). In the light of the above comments, this situation is seemingly paradoxical. Furthermore, there are intense efforts to produce organisms and synthetic catalysts that cycle H₂ without interference from O₂. We define hydrogenases that exhibit catalytic activity in the presence of O₂ as being “O₂ tolerant” (10). The [NiFe] hydrogenases expressed by chemolithoautotrophic Ralstonia species fall into this special category (17). For example, electrochemical experiments have shown that although the membrane-bound hydrogenase (MBH) enzymes from Ralstonia eutropha H16 (Re H16) and Ralstonia metallidurans (Rm

The abbreviations used are: MBH, membrane-bound hydrogenase; PFV, protein film voltammetry; Re, R. eutropha; RH, H₂-sensing, regulatory hydrogenase; Rm, R. metallidurans; SH, NAD-reducing hydrogenase; SHE, standard hydrogen electrode.

5 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
CH₃₄) react rapidly and reversibly with O₂, substantial H₂ oxidation activity remains even in air (9, 18). A practical demonstration of this O₂ tolerance was provided by the construction of an H₂ fuel cell employing Rm CH₃₄ MBH as the anode catalyst and laccase (a multicopper oxidase catalyzing the clean four-electron reduction of O₂ to water) at the cathode. With this device it was possible to produce sufficient electricity from a quiescent atmosphere of just 3% H₂ in air to power a wristwatch for over 24 h (19).

No crystal structure has yet been obtained for any O₂-tolerant hydrogenase. IR spectroscopic experiments suggest that Re H₁₆ MBH has the same coordination arrangement of CO and CN⁻ ligands at the active site as the standard O₂-sensitive [NiFe] hydrogenases (20). An amino acid sequence comparison reveals that Re H₁₆ MBH shares only ~40% identity with standard [NiFe] hydrogenases. However, the amino acids closest to the Re H₁₆ MBH Ni-Fe active site are identical to those in the O₂-sensitive periplasmic hydrogenase from D. gigas, for which a structure has been determined (4). The nearest non-conserved residues to the active site are (according to D. gigas hydrogenase numbering) tyrosine 70 and valine 71, which correspond to glycine 80 and cysteine 81 in Re H₁₆ MBH (Fig. 1B).

To probe whether these specific residues confer O₂ tolerance, a series of exchanges via site-directed mutagenesis was introduced into the Re H₁₆ MBH.

Experiments on the H₂-sensing hydrogenases (RH) from Re H₁₆ and Rhodobacter capsulatus (21, 22) have provided evidence that O₂ tolerance arises from the bulky residues isoleucine and phenylalanine restricting access of O₂ through a narrow gas channel to the active site. In Re H₁₆ MBH, the corresponding residues are valine 77 and leucine 125. To probe whether introducing bulky residues into the MBH further enhances the O₂ tolerance of the enzyme, the residues found in the sensory hydrogenases were introduced into Re H₁₆ MBH by genetic engineering.

The effects of these mutations were investigated in terms of cell growth characteristics and the catalytic properties of the isolated enzymes. The isolated enzymes were studied electrochemically using the suite of techniques known as protein film voltammetry (PFV), which has proved to be very useful in studying hydrogenases from various organisms (9, 10, 20, 23, 24). The enzyme is adsorbed onto an electrode as a sub-monolayer film such that the electrode replaces physiological electron donors and acceptors. Catalytic currents report directly on enzyme activity under conditions of controlled potential (driving force). Using PFV, activity can be measured even under aerobic conditions (18), in which soluble electron donors would be oxidized by O₂.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—The bacterial strains and plasmids and the primers used in this study are listed in Table 1 and Table 2, respectively. Escherichia coli JM109 (25) was used as host in standard cloning procedures, and E. coli S17-1 (26) served as a donor in conjugative transfers. Both Re H₁₆ and Rm CH₃₄ are wild-type strains, and strains carrying the letters HF are derivatives of Re H₁₆.

For purification of the Re MBH variants, we used the plasmid pGE636 carrying the complete MBH operon with a hoxK gene fused with a Streptag II sequence at its 3’ end (27). An equiva-
TABLE 1  
Bacterial strains and plasmids used in this study

| Strains or plasmids | Relevant characteristic(s) | Source or Ref. |
|---------------------|----------------------------|---------------|
| **R. metallidurans** |                            |               |
| CH34                | Wild type                  | DSM2839       |
| **R. eutropha**     |                            |               |
| H16                 | Wild type; MBH+, SH+, RH+, Hox+ | DSM 428, ATCC 17699 |
| pCH1237             |                            | 29            |
| pCH1240             |                            | 29            |
| pCH1241             |                            |               |
| pCH1242             |                            |               |
| pCH1243             |                            |               |
| **E. coli**         |                            |               |
|JM109                | F′ tra336 lacZ ΔlacZ1515 proA B′/e14 (MrA') Δ(lac-proAB) thi gyrA96 (Nal') | 25            |
| S17-1               | Tra' recA pro thi hsdR, chr: RP4-2 | 26            |

**Plasmid**

| Litmus 28           | Ap' lacZ', ColE1 ori          | New England Biolabs |
|---------------------|-------------------------------|---------------------|
| pEDY309             | RK2 ori, Tc', Mob'           | 28                  |
| pCH785              | 21.56-kbp SpeI-XbaI fragment carrying the Re H16 MBH operon in Litmus28 (-SacI) | 29                  |
| pLO6                | MBH overexpression plasmid (pEDY309 derivative carrying the MBH operon) | 29                  |
| pH1351              | 8.96-kbp PstI-Ecl136II fragment carrying hoxKGZMLQGRTV in Litmus28 (-KpnI) | 27                  |
| pH1353              | 9.00-kbp PstI-Ecl136II fragment carrying hoxKGZMLQGRTV with a Strepl II coding sequence fused to the 3′ end of hoxK in Litmus28 (-KpnI) | 27                  |
| pGE636              | 21.60-kbp SpeI-XbaI fragment carrying the Re H16 MBH operon with a Strepl II coding sequence fused to the 3′ end of hoxK in pEDY309 | 27                  |
| pLO2                | Km', sacI, RP4 oriT, ColE1 ori | 30                  |
| pCH1229             | 4.54-kbp MfeI (Klenow-treated)-PstI fragment from pCH1351 in Ecl136II-PstI digested Litmus28 |               |
| pCH1237             | 4.39-kbp XbaI digested PCR fragment (with primers 638 and 639 on pCH1229) | This study         |
| pCH1266             | 2.94-kbp HindIII-BsrGI digested PCR fragment (with primers 641 and 642 on Rm CH34 genomic DNA) in pCH1237 | This study         |
| pCH1267             | 4.40-kbp SpeI-AsISI fragment from pCH1266 in pCH1351 | This study         |
| pCH1268             | 9.02-kbp SpeI-Ecl136II fragment from pCH1267 in pCH785 | This study         |
| pGE636              | 21.57-kbp SpeI-XbaI fragment from pCH1268 in pEDY309 | This study         |
| pCH1269             | 0.62-kbp BssHII fragment from pCH1266 in Litmus28 | This study         |
| pCH1294             | 3.48-kbp RsrII digested PCR fragment (with primers 653 and 654 on pCH1269) | This study         |
| pCH1295             | 0.41-kbp PspOMI-Sall fragment from pCH1294 in pCH1266 | This study         |
| pCH1296             | 4.44-kbp SpeI-AsISI fragment from pCH1295 in pCH1351 | This study         |
| pCH1297             | 9.06-kbp SpeI-Ecl136II fragment from pCH1296 in pCH785 | This study         |
| pGE621              | 21.60-kbp SpeI-XbaI fragment from pCH11297 in pEDY309 | This study         |
| pCH1234             | 0.69-kbp AccD5I-Bmg81I fragment from pCH1351 in Accl51I-EcoRV-digested Litmus28 | This study         |
| pGE610              | Derivative of pGE636 (hoxG C81A, TGT→GGG) | This study         |
| pGE611              | Derivative of pGE636 (hoxG C81S, TGT→TGG) | This study         |
| pGE612              | Derivative of pGE636 (hoxG V77I, TGT→ATC) | This study         |
| pGE613              | Derivative of pGE636 (hoxG V77I, TGT→ATC) | This study         |
| pGE614              | Derivative of pGE636 (hoxG L125F, CTG→TTC) | This study         |
| pGE622              | Derivative of pGE636 (hoxG V77I, TGT→ATC, L125F, CTG→TTC) | This study         |
| pGE639              | Derivative of pGE636 (hoxG G80Y, GGT→TAC) | This study         |
| pGE640              | Derivative of pGE636 (hoxG C81V, TGT→GGT) | This study         |
| pGE641              | Derivative of pGE636 (hoxG G80Y, GGT→TAC, C81V, TGT→GGT) | This study         |

lent plasmid for overproduction of the MBH from Rm CH34 was constructed as follows. A HindIII restriction site within the coding region for the leader sequence of HoxK and a BsrGI site downstream of hoxG were introduced by inverse PCR with primers 638 and 639 using pCH1229 as a template. The 4.39-kbp amplificate was digested with XbaI and re-ligated, resulting in pCH1237. A 2.94-kbp PCR fragment, amplified with primers 640 and 641 on Rm CH34 genomic DNA, was digested with HindIII-BsrGI and inserted into pCH1237 resulting in pCH1266. Plasmid pCH1266 was cut with Spel and AsISI, and

Oxygen-tolerant H₂ Oxidation

JANUARY 2, 2009 • VOLUME 284 • NUMBER 1

JOURNAL OF BIOLOGICAL CHEMISTRY 467
from the L125F derivative was transferred into the previously constructed V77I derivative of pCH1234 yielding a hoxG mutant fragment encoding a V77I/L125F exchange. From all pCH1234 derivatives carrying the hoxG mutations, a 0.68-kbp Acc651-BstZ171 fragment was transferred into pCH1351 and pCH1353. From the pCH1351 derivatives 4.82-kbp Sall-PstI fragments were cloned into pLO2, yielding plasmids that were used for introduction of the mutations into Re HF388 by double homologous recombination as described previously (30). The resulting strains HF681-HF740 are listed in Table 1. From the pCH1353 derivatives, 9.06-kbp Spel-Ecli136II fragments were transferred into pCH785 and, from there, as 21.60-kbp Spel-XbaI fragments into pEDY309. The resulting plasmids pGE610-614, pGE622, and pGE639-641 were transferred into Re HF631 by conjugation. This allowed the overproduction and purification of the MBH variants via a Streptag II fused to the C terminus of the small subunit HoxK. All PCR amplificates were verified by sequencing.

Media and Growth Conditions—Ralstonia strains were grown in Luria broth medium containing 0.25% (w/v) sodium chloride (LSLB) or in mineral salts medium. Synthetic media for heterotrophic growth of Re H16 and Rm CH34 contained 0.4% fructose (FN medium) or 0.2% fructose and 0.2% glycerol (FGN medium) (31). Lithoautotrophic and mixotrophic (H2-supplemented heterotrophic) cultures were grown in mineral salts medium without any carbon source or in FGN medium, respectively, under an atmosphere of H2, CO2, and O2 (8:1:1, v/v), unless otherwise indicated. Cell cultures were grown for 48 h at 120 rpm and 30 °C until they reached an OD436 of ~10, when cultivated heterotrophically, or ~20, when cultivated lithoautotrophically. Cells were harvested by centrifugation (5000 × g at 4 °C for 20 min), washed with phosphate buffer (100 mM Tris/HCl, pH 8.0, 150 mM NaCl), and re-centrifuged. The resulting cell pellet was frozen in liquid N2 and stored at −80 °C. Sucrose-resistant segregants of originally sacB-harboring strains were selected on LSLB plates containing 15% (w/v) sucrose (30). Strains of E. coli were grown in LB medium (32). Solid media contained 1.5% (w/v) agar. The following antibiotics were used: kanamycin (350 μg ml−1) and tetracycline (15 μg ml−1) for Re, and kanamycin (25 μg ml−1), tetracycline (15 μg ml−1), and ampicillin (100 μg ml−1) for E. coli.

MBH Purification—Cell pellets were resuspended in resuspension buffer (100 mM Tris/HCl, pH 8.0, 150 mM NaCl), 4 ml per 1 g of cells (wet weight), containing protease inhibitor mixture (Complete EDTA-free protease inhibitor mixture, Roche Applied Science) and DNase I. The cell suspension was subsequently disrupted in a French pressure cell (Constant Cell Disruption Systems or SLM Aminco). The resulting crude extract was treated by sonication (2 min, level 2.5, 75%, Branson Sonifier), and the membrane and soluble fractions were separated by ultracentrifugation (100,000 × g at 4 °C for 60 min). The brownish membranes were removed, homogenized in an appropriate volume of washing buffer (resuspension buffer + 1 mM EDTA), and ultracentrifuged again (100,000 × g at 4 °C for 30 min). For MBH purification, the membrane proteins were solubilized by adding washing buffer containing Triton X-114 at a final concentration of 2% w/v and subsequently stirring on
ice for 1.5 h. After ultracentrifugation (100,000 × g, 4 °C, 20 min), the supernatant, containing the solubilized membranes, was loaded on Strept-Agarose Superflow columns (IBA, Göttingen, Germany; 1-ml bed volume for up to 25 ml of solubilized membrane extract). The columns were washed with 12 column volumes of washing buffer, and proteins were eluted with 6× 0.5 ml of elution buffer (washing buffer + 5 mM desethylbiotin). MBH-containing fractions were pooled and concentrated using a centrifugal filter device (Amicon Ultra-15 (PL-30), Millipore). The buffer was changed twice by adding 10 ml of storage buffer (50 mM Tris/HC1, pH 8.0, 50 mM NaCl, 1 mM EDTA, 20% glycerol) and subsequent recentrifugation. Aliquots of the resulting concentrate were frozen in liquid N2 and stored at −80 °C. Protein concentrations were determined by the Bradford method (34).

### Electrochemical Measurements of H2 Oxidation Activity

Electrochemical experiments were carried out using an electrochemical cell incorporating a Pt wire counterelectrode. In experiments to determine $K^\text{MH}_M$ of H2-saturated temperature-equilibrated buffer solution (of the same composition to that already in the cell) were injected into the cell solution. Concentrations of O2 or H2 in solution were calculated using the corresponding Henry’s Law constant. In analyzing results from O2 injection experiments, currents were corrected for film loss and normalized by fitting to the exponential curve given in Equation 1,

$$i_t = (i_0 - i_\infty) \exp \left( \frac{t_0 - t}{\tau} \right) + i_\infty$$

(Eq. 1)

in which the time (t) dependence of the current, $i_t$, is described in terms of the current at the start $(t_0)$ of the data to be fitted $(i_0)$, the time constant for film loss ($\tau$), and the limiting current to which the exponential tends $(i_\infty)$.

### RESULTS

**New Protocol for Purification of MBH Proteins from Re H16 and Rm CH34**—In Re H16 the genes coding for the MBH subunits, specific accessory proteins, a set of Hyp proteins, and the H2-sensing apparatus are clustered in a single operon (Fig. 2), which maps on megaplasmid pHG1 (29, 39, 40). The gene organization of the Rm CH34 MBH gene cluster, which is located on chromosome 1 (DOE Joint Genome Institute), resembles that of Re H16 with few exceptions (Fig. 2). The hoxZ gene, encoding a b-type cytochrome, is embedded between hupE encoding a putative nickel transporter and a second open reading frame, with unknown function, annotated as vapi. Unlike the situation in Re H16, the Rm CH34 MBH cluster does not harbor an hupX gene or the response regulator gene hoxA. However, both genes are constituents of a gene cluster encoding the soluble, NAD-reducing [NiFe] hydrogenase, which is located at a different site on chromosome 1 in Rm CH34 (DOE Joint Genome Institute).

To obtain pure hydrogenase for biochemical and electrochemical studies, we exploited an MBH overexpression system repeatedly applied and withdrawn from the electrode surface over a period of 10 s. The electrode was then placed in enzyme-free buffered electrolyte so that all enzyme molecules addressed in the experiments were subjected to the same regime of strict potential control. In all experiments the electrode was rotated at a constant rate (2500–6000 rpm) to provide efficient supply of substrate and removal of product. For experiments carried out at low levels of H2, the electrode tip was gently polished with damp cotton wool to lower the enzyme coverage to minimize the effect of limiting transport of H2 to the electrode. The method is sensitive to extremely low levels of H2 (18) and has a very small sample requirement; determination of an average value for $K^\text{MH}_M$ from >12 repeated experiments can be achieved using just 2 µg of pure protein.

Experiments were performed in phosphate buffer (50 mM; Sigma) containing NaCl (100 mM; Fisher) and titrated to the desired pH at the experimental temperature. All solutions were prepared using purified water (Millipore; 18.2 meq/ohm-cm). Experiments were performed under gas atmospheres of H2 (Premier Grade, Air Products), 1% H2 in N2 (Air Products), or N2 (BOC gases). To measure inhibition by O2, known volume fractions of O2 (Air Products) were injected into the headspace of the electrochemical cell. In experiments to determine $K^\text{MH}_M$, aliquots of H2-saturated temperature-equilibrated buffer solution (of the same composition to that already in the cell) were injected into the cell solution. Concentrations of O2 or H2 in solution were calculated using the corresponding Henry’s Law constants. In analyzing results from O2 injection experiments, currents were corrected for film loss and normalized by fitting to the exponential curve given in Equation 1.

$$i_t = (i_0 - i_\infty) \exp \left( \frac{t_0 - t}{\tau} \right) + i_\infty$$

(Eq. 1)

in which the time (t) dependence of the current, $i_t$, is described in terms of the current at the start $(t_0)$ of the data to be fitted $(i_0)$, the time constant for film loss ($\tau$), and the limiting current to which the exponential tends $(i_\infty)$.
Oxygen-tolerant \( \text{H}_2 \) Oxidation

**FIGURE 3.** Purification of \( \text{Re} \) H16 and \( \text{Rm} \) CH34 MBH proteins. Proteins from different purification steps were separated on a 12% SDS-polyacrylamide gel and visualized by Coomassie Brilliant Blue staining. \( \text{SE} \), soluble extract (20 \( \mu \)g of protein); \( \text{SME} \), soluble membrane extract (20 \( \mu \)g); \( \text{FT} \), flow through from \( \text{Strep}-\text{Tactin Superflow} \); \( \text{EL} \), eluate after \( \text{Strep}-\text{Tactin Superflow} \). (Fig. 2). Representative results of the purification procedure using \( \text{Strep}-\text{Tactin affinity chromatography} \) are given in Fig. 3 and Table 3 for both MBH proteins. Typically, 60 g (wet weight) of cells harboring the MBH overproduction plasmids yielded \( \sim 10 \) mg of nearly homogeneous enzyme with a specific activity of \( \sim 50 \) units mg\(^{-1}\) (Table 3).

Specific activities were routinely obtained in the range 20–120 units mg\(^{-1}\) protein. Using the new purification protocol, we confirmed an earlier observation that the \( \text{O}_2 \) tolerance of the \( \text{Ralstonia} \) MBH proteins, amino acid residues in the vicinity of the active site that are unique to this class of hydrogenases were exchanged. In \( \text{Re} \) H16 MBH, Gly-80 and Cys-81 were exchanged for amino acid residues found at equivalent positions in \( \text{O}_2 \)-sensitive hydrogenases, as indicated in Fig. 1B. This resulted in the series of exchanges G80Y, C81A, C81V, C81S, C81T, and G80Y/C81V, which were introduced into the large subunit (HoxG) of \( \text{Re} \) H16 MBH via site-directed mutagenesis. In addition, Val-77 and Leu-125, which are constituents of the postulated gas channel, were exchanged for bulkier residues, resulting in the exchanges V77I, L125F, and V77I/L125F (as in the RH enzymes from \( \text{Re} \) H16 and \( \text{Rhodobacter capsulatus} \)). To elucidate the origin of the \( \text{O}_2 \) tolerance of the \( \text{Ralstonia} \) MBH proteins, amino acid residues in the vicinity of the active site that are unique to this class of hydrogenases were exchanged. In \( \text{Re} \) H16 MBH, Gly-80 and Cys-81 were exchanged for amino acid residues found at equivalent positions in \( \text{O}_2 \)-sensitive hydrogenases, as indicated in Fig. 1B. This resulted in the series of exchanges G80Y, C81A, C81V, C81S, C81T, and G80Y/C81V, which were introduced into the large subunit (HoxG) of \( \text{Re} \) H16 MBH via site-directed mutagenesis. In addition, Val-77 and Leu-125, which are constituents of the postulated gas channel, were exchanged for bulkier residues, resulting in the exchanges V77I, L125F, and V77I/L125F (as in the RH enzymes from \( \text{Re} \) H16 and \( \text{Rhodobacter capsulatus} \))), to determine whether the MBH became more tolerant to \( \text{O}_2 \).

To test the effect of amino acid alterations on lithoautotrophic growth of \( \text{Re} \) H16 in the presence of high \( \text{O}_2 \) concentrations, the strains were cultivated in liquid medium under an \( \text{H}_2 \) atmosphere containing either 5 or 20% \( \text{O}_2 \). The respective growth rates, derived from the logarithmic growth phase, were determined and are summarized in Table 4, as well as MBH activity and protein stability in the membrane.

Wild-type \( \text{Re} \) H16 responds to the higher \( \text{O}_2 \) concentration with an \( \sim 2 \)–fold lower growth rate (0.045 h\(^{-1}\) compared with 0.079 h\(^{-1}\) at 5% \( \text{O}_2 \)) as determined by optical density measure-
Oxygen-tolerant $H_2$ Oxidation

### TABLE 3
Representative data derived from the purification procedures for Re H16 MBH and Rm CH34 MBH

| Strain       | Volume  | Protein | Total protein | Relative MBH activity | MBH stability in membrane |
|--------------|---------|---------|---------------|-----------------------|--------------------------|
|              | ml      | mg ml$^{-1}$ | mg | units mg$^{-1}$ | %             |
| Re H16 MBH purification |         |         |               |                       |                          |
| Solubilized membrane extract | 70.0 | 4.1 | 290 | 12.9 | 3740 | 100 | 1 |
| Eluate after StSF | 0.30 | 31.4 | 9.4 | 48.1 | 453 | 12.1 | 3.7 |
| Rm CH34 MBH purification |         |         |               |                       |                          |
| Solubilized membrane extract | 81.5 | 5.9 | 483 | 5.6 | 2696 | 100 | 1 |
| Eluate after StSF | 0.44 | 26.1 | 11.5 | 54.6 | 626 | 23.2 | 9.8 |

*StSF indicates Streptactin affinity chromatography.*

### TABLE 4
Characteristics of MBH mutant strain with amino acid exchanges close to the MBH active site

| Strain       | Growth rates (h$^{-1}$) on $H_2$/$O_2$, and $CO_2$ | Relative MBH activity | MBH stability in membrane |
|--------------|---------------------------------------------------|-----------------------|--------------------------|
|              | 5% $O_2$ 20% $O_2$                                 |                       |                          |
| Wild type    | 0.079 0.045                                       | 100                   | +                        |
| G80Y         | No growth                                        | 0                     | (+)†                    |
| G80Y/C81V    | No growth                                        | 0                     | (+)†                    |
| C81A         | 0.069 0.021                                       | 100                   | +                        |
| C81S         | 0.078 0.046                                       | 100                   | +                        |
| C81T         | 0.077 0.027                                       | 75                    | +                        |
| C81V         | 0.081 0.036                                       | 100                   | +                        |
| V77I         | 0.069 0.018                                       | 60                    | +                        |
| L125F        | 0.045 0.004                                       | 100                   | +                        |
| V77L/L125F   | No growth                                        | 30                    | +                       |

* Lithoautotrophic growth was tested in liquid cultures under a gas mixture of 70% $H_2$, 15% $N_2$, 5% $O_2$, 10% $CO_2$, or 70% $H_2$, 20% $O_2$, 10% $CO_2$. When the electrode, modified with a film of hydrogenase, was immersed in electrolyte solution containing $H_2$, the catalytic conversion was observed.*

### Affinity for $H_2$ in Air, Defining an O$_2$ Tolerance Factor—We next used PFV to measure the ability of the purified wild-type and mutant hydrogenases to oxidize $H_2$ in the presence of $O_2$. When the electrode, modified with a film of hydrogenase, was immersed in electrolyte solution containing $H_2$, the catalytic conversion was observed directly on the activity of the enzyme. An electrode with adsorbed MBH as described (see “Experimental Procedures”) was placed in a sealed cell with gas flowing through the headspace and was rotated at 4500 rpm. Electrocatalytic $H_2$ oxidation was observed for films of wild-type and all mutant MBH enzymes with the exception of the G80Y and G80Y/C81V variants, for which no current was observed (supplemental Fig. S2). Maximum catalytic activity was attained at pH 5.5, consistent with photometric measurements (supplemental Fig. S1). For measurements of $K_2O_{Happ}$, the electrode was poised at $-8$ mV versus SHE (as indicated in supplemental Fig. S2), a potential sufficiently positive to give a detectable $H_2$ oxidation current and minimize reduction of $O_2$ at unmodified regions of the graphite, yet sufficiently negative to avoid anaerobic inactivation (18). A typical experiment for the Re H16 MBH (wild type) is shown in Fig. 4. First, $H_2$ (100%, corresponding to 0.8 mm in the electrolyte solution) was flushed through the headspace of the cell to obtain the background current. Aliquots of $O_2$ (1 ml) were then injected into the headspace of the cell (prior to this, the carrier gas flow was halted to prevent the $O_2$ being immediately flushed out), giving 20% $O_2$ in the headspace corresponding to a solution concentration of 0.3 mm at 30 °C. The current was allowed to stabilize after each injection. We note that relatively high activity is retained even at 0.3 mm $O_2$. After the last injection, the cell was again flushed with $H_2$ to remove $O_2$ from the cell. The data were corrected for film loss by extrapolating an exponential fit to the anaerobic data points, as described under “Experimental Procedures.” A plot of
Oxygen-tolerant H₂ Oxidation

![Graph showing the effect of H₂ concentration on the current](image)

**FIGURE 4. Experiment to determine K_{app}^{O_2} by stepwise gas injections.** The electrode, modified with Re H16 MBH enzyme, was held under 100% H₂ at −8 mV versus SHE, pH 5.5, 30 °C, rotating at 4500 rpm. Aliquots of O₂ were injected into the headspace of the cell, causing the current to fall, before the cell was flushed with 100% H₂. The current versus time trace was corrected for film loss by fitting the anaerobic data points to an exponential decay. The inset shows a plot used to calculate K_{0}^{app}.

**TABLE 5**

| Enzyme                  | K_{M}^{O_2} | K_{M}^{O_2} | K_{M}^{O_2} |
|-------------------------|-------------|-------------|-------------|
| Wild-type Rm CH34 MBH   | 730         | 0.57        |             |
| Wild-type Re H16 MBH    | 1000        | 6.1         |             |
| Re H16 C81A MBH         | 1400        | ~300        |             |
| Re H16 C81S MBH         | 1200        | 44          |             |
| Re H16 C81T MBH         | 870         | 11          |             |
| Re H16 C81V MBH         | 1200        | 3.9         |             |
| Re H16 V71 MBH          | 480         | 8.1         |             |
| Re H16 L125F MBH        | 400         | 69          |             |
| Re H16 V71L125F MBH     | 260         | ~800        |             |

*All values of K_{M}^{O_2} were determined, pH 5.5 and 30 °C, with an electrode potential of −8 mV versus SHE, in 800 μM H₂.

*All values of K_{M}^{O_2} were determined at a potential of −108 mV versus SHE, at pH 5.5 and 30 °C and are quoted to two significant figures.

*This value was determined under 8 μM H₂.

*These values were determined at 30 °C.

---

Using this method, Léger et al. (24) studied the O₂-sensitive [NiFe] hydrogenase from *D. fructosovorans*. In contrast to solution assays in which a soluble electron acceptor is employed, the potential experienced by the enzyme is strictly defined in the electrochemical experiment. Using a slight adaptation of this method, in which gas concentrations were determined by the composition of the gas flowing through the headspace of the sealed electrochemical cell, we determined values for K_{M}^{O_2} for Rm CH34 and Re H16 wild-type MBH enzymes as well as the genetic variants of Re H16 MBH. Within the window of electrochemical potential over which H₂ oxidation is observed, we chose a sufficiently negative potential, −0.108 mV versus SHE, to avoid anaerobic inactivation of the Ni-Fe center that occurs at high potential (9). Fig. 5A shows a typical result for Rm CH34 MBH. At a given time, defined as t = 0, a known volume of H₂-saturated buffer (of identical composition and temperature to that already in the electrochemical cell) was injected to give an initial H₂ concentration of 0.4 mM in solution, causing the electrocatalytic current to rise rapidly. The H₂ concentration immediately begins to drop because of the N₂ flow through the cell headspace, and independent experiments confirmed that the drop in H₂ concentration follows an exponential decay, as indicated in Fig. 5A (right axis). The initial current (i_{max}) remains fairly stable for more than 200 s indicating the range of H₂ concentrations over which the enzyme is substrate-satu-

---

Reciprocal current (1/i) against O₂ concentration gave a straight line, with K_{0}^{app}, obtained by dividing the y intercept by the gradient. All calculated values of K_{0}^{app} for both wild-type and mutant MBH enzymes are given in Table 5. Because each O₂ injection expelled an equal volume of H₂ from the headspace, the H₂ concentration was lowered to 80% of the initial level during the experiment, i.e. 0.64 mM in solution. This change in substrate concentration is not significant for most of the MBH enzymes because these H₂ levels are well above their Michaelis constant (K_{M}^{H₂}) for H₂ (see below), but for the C81A and V71L/125F mutants, which have a particularly high K_{M}^{H₂} (gain see below), the values of K_{0}^{app} are likely to be slightly underestimated. For Rm CH34 MBH, K_{M}^{H₂} is sufficiently low that it was possible also to determine K_{0}^{app} under just 1% H₂ (corresponding to 8–6.4 μM H₂ in the cell solution during the experiment), and a value of ~5-fold lower than at 100% H₂ was obtained (Table 5).

**Affinity for H₂ under Anaerobic Conditions**—With the exception of the C81A mutant, there appears to be a general correlation between the rate of lithoautotrophic cell growth and K_{0}^{app} of the isolated MBH enzyme. For the C81A mutant, however, the isolated MBH enzyme has a high K_{0}^{app} but cell growth is retarded relative to wild type. Coupled with the observation that K_{0}^{app} for Rm CH34 MBH is diminished at a lower H₂ concentration, this led us to examine K_{M}^{H₂} for the isolated MBH enzymes, to see whether affinity for H₂ contributes to O₂ tolerance.

To determine values of K_{M} for H₂ oxidation (K_{M}^{H₂}) by wild-type MBH from Re H16 and Rm CH34, we initially used a Clark-type oxygen electrode to monitor H₂ concentration as a function of time with methylene blue (0.6 mM) as electron acceptor (see under “Experimental Procedures”). We obtained K_{M}^{H₂} values of 14.9 μM for Re H16 MBH and 9.1 μM for Rm CH34 MBH. These values are probably too high to reflect the physiological requirement for oxidation of trace H₂. Overestimation of K_{M}^{H₂} in these assays is likely because a K_{M} value of 1.3 mM for methylene blue has been determined previously for Re H16 MBH (41, 42).

We therefore adopted a different approach and used a modification of the direct electrochemical method described by Léger et al. (24), for determining affinity constants for enzymes reacting with gaseous substrates. This concept involves introducing a gas and monitoring the change in catalytic current as this gas is flushed out of the electrochemical cell by a stream of carrier gas. Values of K_{M}^{H₂} are extracted by analysis of the current time profiles described by Equation 2, which incorporates the Michaelis-Menten equation and the exponential profile for loss of H₂ from solution. The H₂ concentration at zero time (t = 0) is denoted C_{H₂}; i_{max} is the maximum catalytic current corresponding to substrate saturation, and τ is the time constant for the removal of substrate by the carrier gas.

\[
i_t = \frac{i_{max}}{1 + \frac{K_{M}^{H₂}}{C_{H₂}(0)} \exp(t/\tau)} \quad (Eq. 2)
\]
Controlled-potential gas-transient experiments were designed to measure $K_{m}^{H_2}$ for Rm CH34 MBH (A), Re H16 MBH (B), and Re V77I/L125F MBH (C). The experiments shown in A and B were conducted as follows. At time \( t = 0 \), temperature-equilibrated $H_2$-saturated buffer was injected into the cell solution to give an initial concentration, $[H_2]_0$, of 0.4 mM. The $H_2$-oxidation current response as a function of time is shown as a function trace. The initial plateau in the current, reflecting the initial concentration of $H_2$ in solution as a function of time data (Fig. 5A, dashed line) allows determination of $K_{m}^{H_2}$. The time at which the current is equal to $i_{max}/2$ is attained. This slow rise in activity is not simply because of gas mixing (as evidenced by the long time constant for this phase; the current continues to rise for $\sim 100$ s). This rise is also noticeable for Rm CH34 MBH, although it is far less pronounced. Experiments to probe this feature are underway. The initial plateau in the current, reflecting the range of $H_2$ concentrations over which the enzyme remains substrate-saturated, is shorter for Re H16 than Rm CH34 MBH, indicating that the former has a lower affinity for $H_2$. This is reflected in the value of $K_{m}^{H_2}$ obtained for Re H16 MBH, 6.1 $\mu$M. Values of $K_{m}^{H_2}$ were also determined at 20°C (Table 5).

This procedure was applied to the series of mutants of Re H16 MBH (Fig. 5B). In this case there was always a short delay following the injection of $H_2$-saturated buffer before the maximum current, $i_{max}$, was attained. This slow rise in activity is not simply because of gas mixing (as evidenced by the long time constant for this phase; the current continues to rise for $\sim 100$ s). This rise is also noticeable for Rm CH34 MBH, although it is far less pronounced. Experiments to probe this feature are underway. The initial plateau in the current, reflecting the range of $H_2$ concentrations over which the enzyme remains substrate-saturated, is shorter for Re H16 than Rm CH34 MBH, indicating that the former has a lower affinity for $H_2$. This is reflected in the value of $K_{m}^{H_2}$ obtained for Re H16 MBH, 6.1 $\mu$M. Values of $K_{m}^{H_2}$ were also determined at 20°C (Table 5).

Oxygen-tolerant $H_2$ Oxidation

First, voltammograms of all Ralstonia MBH enzymes (see supplemental Fig. S2) suggest heterogeneity; this is observable as an additional minor local current maximum in the region between $-0.1$ and $-0.2$ V. The origin of this feature is not known and is currently being investigated. Second, Ralstonia MBH enzymes undergo anaerobic inactivation at high potentials, and there is no region of potential available in which the catalytic current (activity) is independent of potential. This is more pronounced for Re H16 MBH (and most of the mutants) than for Rm CH34 MBH (supplemental Fig. S2). Third, there is the possibility of back diffusion of low levels of $H_2$ into the cell from the glovebox; one of the assumptions underlying Equation 2 is that the $H_2$ concentration in the cell tends to 0 over time. Finally, mass transport limitation is unavoidable at extremely low levels of $H_2$, even at high rotation rates, and this is not accounted for in Equation 2.

Analogous experiments were performed for wild-type Re H16 MBH (Fig. 5B). In this case there was always a short delay following the injection of $H_2$-saturated buffer before the maximum current, $i_{max}$, was attained. This slow rise in activity is not simply because of gas mixing (as evidenced by the long time constant for this phase; the current continues to rise for $\sim 100$ s). This rise is also noticeable for Rm CH34 MBH, although it is far less pronounced. Experiments to probe this feature are underway. The initial plateau in the current, reflecting the range of $H_2$ concentrations over which the enzyme remains substrate-saturated, is shorter for Re H16 than Rm CH34 MBH, indicating that the former has a lower affinity for $H_2$. This is reflected in the value of $K_{m}^{H_2}$ obtained for Re H16 MBH, 6.1 $\mu$M. Values of $K_{m}^{H_2}$ were also determined at 20°C (Table 5).

This procedure was applied to the series of mutants of Re H16 MBH (Fig. 5 and supplemental Fig. S3). All values of $K_{m}^{H_2}$ are given in Table 5. The $H_2$ oxidation current versus time profile for $H_2$ is flushed out of solution gives an intermediate qualitative indication of relative affinity for $H_2$, provided experimental conditions, including gas flow rate and electrode rotation rate, are held constant. In experiments on some mutants, in particular C81A, L125F, and V77I/L125F, a current plateau was not attained following injection of $H_2$-saturated solution, indicating that these variants are not substrate-saturated at 0.4 mM $H_2$.
Oxygen-tolerant H₂ Oxidation

To achieve a higher initial H₂ concentration (C\(_{H₂}(0)\)), H₂ was flushed through the headspace of the cell to give an initial concentration of 0.8 mM, and at the time designated \( t = 0 \), the gas flow was changed to N\(_₂\) to flush out H₂. Experiments conducted in this way on the wild-type enzymes confirmed that values of \( K_{M}^{H₂} \) are consistent with those obtained using the injection method.

Qualitative analysis of the current versus time profiles for the C81A and V77I/L125F MBH enzymes (Fig. 5C and supplemental Fig. S3) indicate that these mutants are clearly unsaturated even at 0.8 mM H₂. The Michaelis-Menten model normally considers an enzyme to be saturated at substrate concentrations in excess of 10× \( K_{M}^{H₂} \), and applying this “rule” indicated that \( K_{M}^{H₂} \) is >80 μM for these mutants. Attempts to fit the data for these mutants yielded only approximate values for \( K_{M}^{H₂} \) as \( i_{\text{max}} \) could not be obtained directly from the current versus time profiles; however, as expected, the calculated values are well above 80 μM. The current versus time profile for the L125F mutant indicates that this variant is close to substrate saturation at 0.8 mM H₂, and the value of 69 μM obtained for \( K_{M}^{H₂} \) is consistent with this.

DISCUSSION

We have carried out an integrated physiological, biochemical, and electrochemical analysis of the catalytic properties of the membrane-bound [NiFe] hydrogenases derived from the two prominent aerobic H₂ oxidizers *R. eutropha* H16 and *R. metallidurans* CH34. For hydrogenase purification, overproduction plasmids were used that contain the entire genetic information for synthesis and maturation of catalytically active enzyme (29). The genes encoding the small subunit were equipped with a Streptag II sequence allowing a mild and easy one-step purification of the fully matured, heterodimeric MBH protein. Purification of active CH34 MBH was achieved simply by exchanging the structural genes of *Re* H16 with the respective counterparts from *Rm* CH34. The heterologous production of active [NiFe] hydrogenase has been reported for several examples (43, 44). We have demonstrated here the cross-compatibility of a protease that is normally highly substrate-specific; i.e. the endopeptidase of *Re* H16 MBH was able to process, efficiently, the premature large subunit of *Rm* CH34 MBH. Furthermore, the *Rm* CH34 MBH is entirely compatible with the *Re* H16 maturation apparatus and functionally interacts with the MBH-specific cytochrome b of *Re* H16. These observations are probably because of the fact that the H16 and CH34 MBH subunits share more than 80% identity. However, immunological analysis revealed that the content of the CH34 MBH was lower in the membrane of *Re* H16 than that of the indigenous H16 MBH indicating a less strong connection to the primary electron acceptor, the cytochrome b HoxZ of *Re*. For this reason we did not include the *Re* strain carrying the CH34 MBH in our growth experiments.

In *vivo*, the [NiFe] hydrogenases of aerobic, H₂-oxidizing Knallgas bacteria permanently face challenging conditions. The H₂ concentrations in their habitats are usually extremely low (possibly 1–10 nM (45), equivalent to 1–10 ppm gas fraction), whereas the O₂ concentration is close to the 21% prevailing in the atmosphere. Consequently, the H₂-converting enzymes of Knallgas bacteria must be highly selective for H₂ with a concomitant high tolerance toward O₂, although the origin of this selectivity is unclear. The enzymes clearly do react rapidly with O₂, even though they are not completely inhibited in its presence. (Note that in Fig. 4 the time taken for the current to stabilize at each stage is determined by the gas mixing time following injections of O₂ into the headspace of the cell and not directly into the cell solution.) Therefore, the origin of their O₂ tolerance cannot simply be a physical exclusion of O₂ from the active site. The conserved nature of the amino acid residues around the active site between O₂-sensitive and O₂-tolerant hydrogenases, and the consistent ligation of the iron and nickel atoms, raises interesting questions about the origin of the differences in affinities for H₂ and sensitivity to O₂ of hydrogenases from different organisms.

The term that we refer to as \( K_{O_2}^{(app)} \) should be regarded as giving an empirical measurement of O₂ tolerance (a high \( K_{O_2}^{(app)} \) implies a high O₂ tolerance). In addition to temperature and pH, \( K_{I} \) also depends not only on partial pressure of H₂ but also on electrode potential. In principle, as the partial pressure of H₂ tends toward 0, \( K_{O_2}^{(app)} \) becomes a realistic measurement of the O₂ tolerance that is likely to prevail in *vivo*.

In an early investigation, Schink and Probst (46) presented evidence that O₂ inhibits the *Re* H16 MBH activity in a competitive manner. However, it is most unlikely that inhibition by O₂ is this straightforward as it is now established that O₂ reacts with the Ni-Fe active site in a range of hydrogenases to produce chemically modified states that require reductive re-activation at well defined potentials (9, 47). Reaction of O₂ with standard [NiFe] hydrogenases gives a mixture of Ni-A and Ni-B inactive states. However, in recent spectroscopic investigations on *Re* H16 MBH, reaction with O₂ produced only Ni-B; formation of the O₂-inhibited Ni-A state was not observed under any conditions. This evidence, combined with our electrochemical data, leads to the model shown in Fig. 6. Although O₂ cannot be viewed as a simple competitive inhibitor in the accepted sense, H₂ and O₂ may thus be regarded as “competitive substrates”: the O₂-inactivated forms are recovered by reduction, with the net result that O₂ is reduced (and is therefore, technically, a substrate). The turnover frequency for this “oxidase” activity is likely to be limited by the rate-determining reactivation of Ni-B
that completes the competing cycle. A high affinity for H₂ is likely to keep the MBH enzyme in the productive H₂ oxidation cycle. Experiments have shown that the Ralstonia MBH enzymes are reductively reactivated much more rapidly, and at a higher potential, than standard O₂-sensitive [NiFe] hydrogenases such as the enzymes from A. vinosum and D. gigas (9, 48). It follows therefore that K_{H2}^{O2} reflects several complex kinetic factors. Detailed experiments to measure the potential dependence of reactivation rates for the Ralstonia enzymes following O₂ exposure are currently underway.

The values of K_{H2}^{O2} are too low to be determined accurately for standard O₂-sensitive [NiFe] hydrogenases. For example, under 100% O₂, the enzyme from D. fructosovorans was found to be completely inhibited by just 4 μM O₂ at +190 mV (24), and A. vinosum [NiFe] MBH is completely inhibited by 6 μM O₂ at +142 mV (20). In this study, we have determined apparent inhibition constants (K_{H2}^{O2 (app)}) for Rm CH34 and Re H16 MBH enzymes as 730 and 1000 μM, respectively, at −8 mV, many orders of magnitude higher than observed for standard [NiFe] hydrogenases. For Rm CH34 MBH, K_{H2}^{O2 (app)} was higher at 0.8 mm H₂ than at 8 μM H₂, fully consistent with competition between H₂ and O₂.

It would therefore be anticipated that lowering the affinity for H₂ (i.e. increasing K_{H2}^{O2}) would result in a lower K_{H2}^{O2 (app)}. To determine values of K_{H2}^{O2}, we applied the direct electrochemical method described by Léger et al. (24). Under comparable conditions (30 °C, pH 5.5) to those used to determine K_{H2}^{O2 (app)} Rm CH34 MBH exhibited a K_{H2}^{O2} value of 0.57 μM, whereas K_{H2}^{O2} for the Re H16 MBH is 10-fold higher, at 6.1 μM. Both values are in the range reported for hydrogenases from other aerobic H₂ oxidizers (49–51) and reflect the requirement for Ralstonia species to scavenge trace H₂ from their environment. Aerobic H₂ oxidizers most likely persist at Earth’s average surface temperature of 15 °C. At 20 °C, Rm CH34 MBH displayed a K_{H2}^{O2} of 90 nm (~90 ppm), close to the range (11–83 nm) reported for so-called “soil hydrogenases” that represent the largest sink (~75%) for atmospheric H₂ (52, 53). As the level of H₂ in the lower atmosphere (0.5 ppm (54)) is well below the threshold value required for uptake by Knallgas bacteria (1–2 ppm (55)), it has been speculated that aerobic chemolithothrobes rely upon H₂ that is fermentatively produced in periodically anoxic, submerged soils (56). Consistent with this is the recent observation that oxidation of H₂ at levels down to 10 ppm in air is easily detectable for Rm CH34 MBH (18).

Regarding correlations between K_{H2}^{O2 (app)} and enzyme structure, we note that the closest amino acids to the active site showing significant variations in Re H16 MBH compared with standard O₂-sensitive hydrogenases are Gly-80 and Cys-81. Both residues are more than 4.5 Å away from the metal atoms and therefore are not expected to be directly influential in coordination of the active site or substrates but could be important in structure stabilization or transport of small molecules or ions. A series of mutant proteins targeting these residues was prepared to probe their role in conferring O₂ tolerance on the MBH enzyme. In the absence of structural data, the precise location of these mutations in the protein, and the effect that they exert on the folding of the protein is of course unclear. The substitutions may also have electronic effects at the active site.

Exchange of Gly-80 by Tyr results in an unstable MBH with no activity. Even the introduction of a second mutation (C81V) to fully mimic the structure of D. gigas did not solve this problem. We therefore conclude that Gly-80 plays an indispensable role in Re H16 MBH.

For the mutations that resulted in active MBH, we compared the rate of lithoautotrophic growth with that of wild-type Re H16. Wild-type cultures show slower growth at 20% (v/v) O₂ than at 5% O₂. Substitution of Cys-81 by Ser, Thr, or Val has very little effect on cell growth on H₂ at the lower O₂ concentration (Table 4), and the membrane extracts show H₂ oxidation activity similar to the wild type. Consistent with this, the values of K_{H2}^{O2 (app)} determined for these mutants are within the range ±20% of the value for the wild-type enzyme. The retarded growth of C81T strain at 20% O₂ correlates with the slightly lower K_{H2}^{O2 (app)}. This correlation is more evident for the mutants designed to place bulky residues in the mouth of a putative gas channel through the protein (see below). For these mutants, K_{H2}^{O2 (app)} decreases in the order V77I > L125F > V77I/L125F, correlating with increasing growth retardation relative to wild type under both 5 and 20% O₂.

Results for the C81A mutant are surprising because although K_{H2}^{O2 (app)} is significantly higher than for the wild type, cell growth was retarded slightly at 5% O₂ and substantially at 20% O₂. It was therefore important to determine K_{H2}^{O2} values for C81A and the other isolated mutant MBH enzymes.

For the V77I/L125F and V77I/L125F mutants, a trend in increasing K_{H2}^{O2} correlates with decreasing K_{H2}^{O2 (app)} and greater growth retardation. However, this correlation is not observed for the C81A mutant, which has an extremely high K_{H2}^{O2} (at least 20-fold higher than the wild type) even though K_{H2}^{O2 (app)} is increased relative to wild type. The C81S mutant also exhibits both a higher K_{H2}^{O2} value and a slightly higher K_{H2}^{O2 (app)} compared with wild type, although its cell growth rate remains unaffected.

According to the D. gigas structure, Cys-81 lies in the vicinity of the CO ligand to the active site iron atom. A CO ligand is special because compression of a M-CO bonding arrangement lowers electron density on the metal (M), and amino acid alterations close to the CO can influence the acceptor properties of this ligand, and therefore affect active site reactivity. It is therefore not surprising that exchanging Cys-81 alters the behavior of the MBH.

It has been suggested that specific gas channels allow H₂ and O₂ to access the active site of hydrogenases. Because of the larger size of O₂, constrictions in these channels may affect its transport more than H₂. In support of this hypothesis, the O₂-tolerant H₂-sensing hydrogenases from Re H16 and R. capsulatus have bulky amino acid residues (Phe and Ile) positioned at the end of a putative gas channel, and mutation of these to smaller residues (Leu and Val, respectively) leads to O₂ sensi-
Oxygen-tolerant H₂ Oxidation

tivity (21, 22). In wild-type Re H16 MBH, Leu and Val are present at the equivalent positions, and mutation to Phe and Ile, respectively, was therefore expected to confer increased O₂ tolerance. Interestingly, the double exchange brought about a 100-fold increase of $K_{M}^{H₂}$ relative to the wild-type enzyme, whereas the catalytic activity was only reduced 3-fold, consistent with a restricted gas flow to the catalytic center. However, the mutant proteins were actually more O₂-sensitive than the wild-type protein, as expressed by the 4-fold decrease in $K_{M}^{O₂}$ (app). In a very recent study the same exchange strategy was applied to proposed gas channel of the standard [NiFe] hydrogenase from D. fructosovorans (57). The corresponding V74I/L122F mutant also showed a significant increase in the $K_{M}^{O₂}$ but concomitantly exhibited a 10-fold higher tolerance toward CO (which is a potent inhibitor for the D. fructosovorans wild-type enzyme (57) but not for Re MBH (20)).

The experiments presented in this study have explored several aspects of the issue of “O₂ tolerance” in [NiFe] hydrogenases. Although it is still not possible to directly identify molecular factors that render the MBH enzymes from Ralstonia significantly less O₂-sensitive than corresponding enzymes from other species, a number of conclusions can be drawn. The wild-type MBH enzymes have values of $K_{M}^{H₂}$ at the lower end of the range reported for [NiFe] hydrogenases. The mutants showing substantially elevated $K_{M}^{H₂}$ (C81A and V74I/L122F) exhibited poor growth relative to wild type, indicating that a low $K_{M}^{H₂}$ is an important characteristic for enabling lithoautotrophic growth. All mutations that resulted in a lowered $K_{M}^{O₂}$ (app) also had an elevated $K_{M}^{H₂}$ and were associated with strains that showed impaired growth on H₂ and O₂ indicating that good competition between H₂ and O₂ at the MBH active site is important for lithoautotrophic growth. A correlation between $K_{M}^{H₂}$ and $K_{M}^{O₂}$ (app) might be expected, given that $K_{M}^{O₂}$ is sensitive to H₂ concentration, but this is not always the case (the correlation does not hold for the C81A and C81S mutants).

The O₂ tolerance of Ralstonia MBH enzymes cannot be simply linked to single point mutations in the vicinity of the active site; mutants of Re H16 MBH in which the closest varying residues were exchanged for those found in O₂-sensitive hydrogenase; mutants of Re linked to single point mutations in the vicinity of the active site, indicating that a low $K_{M}^{H₂}$ is an important characteristic for enabling lithoautotrophic growth. All mutations that resulted in a lowered $K_{M}^{O₂}$ (app) also had an elevated $K_{M}^{H₂}$ and were associated with strains that showed impaired growth on H₂ and O₂ indicating that good competition between H₂ and O₂ at the MBH active site is important for lithoautotrophic growth. A correlation between $K_{M}^{H₂}$ and $K_{M}^{O₂}$ (app) might be expected, given that $K_{M}^{O₂}$ is sensitive to H₂ concentration, but this is not always the case (the correlation does not hold for the C81A and C81S mutants).

The O₂ tolerance of Ralstonia MBH enzymes cannot be simply linked to single point mutations in the vicinity of the active site; mutants of Re H16 MBH in which the closest varying residues were exchanged for those found in O₂-sensitive hydrogenases did not show significantly enhanced O₂ sensitivity compared with the wild type. Tolerance to O₂ is clearly a complex factor and is determined by a well-adapted spatial and electronic structure of the active site rather than a simple restriction of diffusion of inhibitory gases such as O₂. Further studies to investigate kinetic and thermodynamic details of the reactions of the active site with H₂ and O₂ are required.

Acknowledgments—We thank Bärbel Friedrich for valuable discussions and suggestions and for generous support. We thank A. Wait for obtaining preliminary data on the affinity of Re C81A MBH for H₂ and for showing that G80Y and G80Y/C81V variants of Re H16 MBH are inactive in electrochemical experiments. We also thank Dr. C. Blanford for helpful discussions. The R. metallidurans CH34 sequence data were produced by the United States Department of Energy Joint Genome Institute (www.jgi.doe.gov/).

REFERENCES
1. Cammack, R., Robson, R., and Frey, M. (eds) (1997) Hydrogen as a Fuel: Learning from Nature, Taylor & Francis Ltd., London
2. Shima, S., and Thauer, R. K. (2007) Chem. Rev. 107, 37–46
3. Fontecilla-Camps, J. C., Volbeda, A., Cavazza, C., and Nicolet, Y. (2007) Chem. Rev. 107, 4237–4303
4. Volbeda, A., Charon, M. H., Piras, C., Hatchikian, E. C., Frey, M., and Fontecilla-Camps, J. C. (1995) Nature 373, 580–587
5. Albracht, S. P. (1994) Biochim. Biophys. Acta 1188, 167–204
6. Surerus, K. K., Chen, M., van der Zwaan, J. W., Rusnak, F. M., Kolk, M., Duin, E. C., Albracht, S. P., and Münck, E. (1994) Biochimie 33, 4980–4993
7. Volbeda, A., Garcin, E., Piras, C., de Lacey, A. L., Fernandez, V. M., Hatchikian, E. C., Frey, M., and Fontecilla-Camps, J. C. (1996) J. Am. Chem. Soc. 118, 12989–12996
8. Pierik, A. J., Roseboom, W., Happe, R. P., Bagley, K. A., and Albracht, S. P. (1999) J. Biol. Chem. 274, 3331–3337
9. Vincent, K. A., Parkin, A., Lenz, O., Albracht, S. P., Fontecilla-Camps, J. C., Cammack, R., Friedrich, B., and Armstrong, F. A. (2005) J. Am. Chem. Soc. 127, 18179–18189
10. Vincent, K. A., Parkin, A., and Armstrong, F. A. (2007) Chem. Rev. 107, 4366–4413
11. Cammack, R., Fernandez, V. M., and Schneider, K. (1986) Biochimie (Paris) 68, 85–91
12. Bleijlevens, B., van Broekhuizen, F. A., De Lacey, A. L., Roseboom, W., Fernandez, V. M., and Albracht, S. P. (2004) J. Biol. Inorg. Chem. 9, 743–752
13. Cammack, R., Belsey, N. A., Lubitz, W., and Armstrong, F. A. (2006) Biochim. Biophys. Acta 176, 69–752
14. Jones, A. K., Lamle, S. E., Pershad, H. R., Vincent, K. A., Albracht, S. P., and Armstrong, F. A. (2003) J. Am. Chem. Soc. 125, 8505–8514
15. Burgdorf, T., Lenz, O., Buhrke, T., van der Linden, E., Jones, A. K., Albracht, S. P., and Friedrich, B. (2005) J. Mol. Microbiol. Biotechnol. 10, 181–196
16. Duche, O., Eiken, S., Dornac, L., and Colbeau, A. (2005) FEBS J. 272, 3899–3908
17. Vincent, K. A., Belsey, N. A., Lubitz, W., and Armstrong, F. A. (2006) J. Am. Chem. Soc. 128, 7448–7449
18. Léger, C., Dementin, S., Bertrand, P., Rousset, M., and Guigliarelli, B. (2004) J. Am. Chem. Soc. 126, 12162–12172
19. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Gene (Amst.) 33, 103–119
20. Simon, R., Priefert, U., and Pühler, A. (1983) Bio/Technology 1, 784–790
21. Schubert, T., Lenz, O., Krauss, E., Volkmann, R., and Friedrich, B. (2007) Mol. Microbiol. 66, 453–467
22. Kleihues, L., Lenz, O., Bernhard, M., Buhrke, T., and Friedrich, B. (2000) J. Bacteriol. 182, 2716–2724
23. Lenz, O., Gleiche, A., Strack, A., and Friedrich, B. (2005) J. Bacteriol. 187, 6590–6595
24. Lenz, O., Schwartz, E., Derrnedde, I., Eitinger, M., and Friedrich, B. (1994) J. Bacteriol. 176, 4385–4393
25. Schwartz, E., Gerischer, U., and Friedrich, B. (1998) J. Bacteriol. 180, 3197–3204
26. Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Har-
