How oxygen reacts with oxygen-tolerant respiratory [NiFe]-hydrogenases

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Abstract

An oxygen-tolerant respiratory [NiFe]-hydrogenase is proven to be a four-electron hydrogen/oxygen oxidoreductase, catalyzing the reaction $2\text{H}_2 + \frac{1}{2}\text{O}_2 = \text{H}_2\text{O}$, equivalent to hydrogen combustion, over a sustained period without inactivating. At least 86% of the H$_2$O produced by *Escherichia coli* hydrogenase-1 exposed to a mixture of 90% H$_2$ and 10% O$_2$ is accounted for by a direct four-electron pathway, whereas up to 14% arises from slower side reactions proceeding via superoxide and hydrogen peroxide. The direct pathway is assigned to O$_2$ reduction at the [NiFe] active site, whereas the side reactions are an unavoidable consequence of the presence of low-potential relay centers that release electrons derived from H$_2$ oxidation. The oxidase activity is too slow to be useful in removing O$_2$ from the bacterial periplasm; instead, the four-electron reduction of molecular oxygen to harmless water ensures that the active site survives to catalyze sustained hydrogen oxidation.

H$_2$ | mass spectrometry | Fe-S cluster

Hydrogenases are enzymes that catalyze the interconversion of H$_2$ and H$^+$ with great efficiency. Containing Fe or Fe and Ni as active metals, they are not only important in biohydrogen production (by fermentative and photosynthetic means) but also provide inspiration for detailed understanding and development of optimal molecular electrocatalysts. The minimal active site motif, common to all hydrogenases, is a low-spin Fe atom coordinated by CO, CN$^-$, and thiolate ligands, a combination expected to be unstable under aerobic conditions. Indeed, most hydrogenases suffer long-term or permanent inactivation when exposed to even traces of O$_2$. It is therefore of special interest that certain [NiFe]-hydrogenases have evolved to sustain H$_2$ oxidation in the continued presence of O$_2$ without inactivation: these enzymes are known as O$_2$-tolerant [NiFe]-hydrogenases.

Most of our current insight into the mechanism of O$_2$ tolerance stems from studies on respiratory membrane-bound [NiFe]-hydrogenases that couple H$_2$ oxidation to reduction of quinones (1–3). These enzymes are localized at the cytoplasmic membrane and project into the periplasmic space. A model proposed for the O$_2$-tolerance mechanism of these [NiFe]-hydrogenases (Fig. 1) is based on the following evidence. Oxygen reacts with O$_2$-tolerant membrane-bound [NiFe]-hydrogenases to form, exclusively, an inactive state known as Ni-B or “ready,” formulated as a Ni(III)-OH$^-$, and an O$_2$-sensitive [NiFe]-hydrogenases react with O$_2$ to give a mixture of states, including ones variously known as “unready” or Ni-A, in which O$_2$ is either only partially reduced (possibly trapped as a peroxide) or has oxygenated atoms of the active site (3–7). The unready states are only reactivated very slowly; consequently, their production removes enzyme from the catalytic pool (8, 9).

The implications are that, to avoid unready states completely, O$_2$-tolerant [NiFe]-hydrogenases have the special capability to provide, very rapidly, the electrons (and necessary protons) required to ensure complete reduction of an O$_2$ molecule each time it attacks. Without high fidelity in this respect, enzyme molecules become progressively inactivated until, finally, no activity remains (9).

Recent studies on the respiratory membrane-bound periplasmic hydrogenases have linked this capability to unique features of the electron relay system within the enzyme.

Fig. 2A shows the structure of an O$_2$-tolerant [NiFe]-hydrogenase known as hydrogenase-1 (Hyd-1), which is produced in *Escherichia coli* (10). Like other respiratory membrane-bound [NiFe]-hydrogenases, it contains a buried [NiFe] catalytic center and FeS clusters that are located in separate α and β subunits. The FeS clusters are positioned in such a way as to provide a long-range electron relay between the [NiFe] site and the protein surface. In terms of quaternary structure, the membrane-extrafibrillar (periplasmic) domain of Hyd-1 is an (αβ)$_2$ homologous heterodimer. Further, as demonstrated by the recent structure of a Hyd-1 variant, the complete enzyme also comprises a labile transmembrane α-helical region housing at least one b-type cytochrome (11).

Unlike the standard, O$_2$-sensitive hydrogenases that contain a conventional [4Fe-4S] cluster in the position proximal to the active site, the O$_2$-tolerant enzymes contain a thus-far unique [4Fe-3S] cluster that is ligated by six rather than four cysteines. As illustrated in Fig. 2B, the unusual structure allows it to undergo two consecutive one-electron transfers at similar potentials—the rapid removal of the second electron (in a proton-coupled reaction) yielding a Fe-N(peptide) bond in a reaction that is (locally at least) electroneutral (1, 2, 10, 12–15). The [3Fe-4S] cluster occupying the medial position in respiratory membrane-bound [NiFe]-hydrogenases also has a higher reduction potential in O$_2$-tolerant hydrogenases (e.g., 190 ± 30 mV at pH 6 in *E. coli* Hyd-1) (15), than in standard hydrogenases (e.g., ~70 mV at pH 7 in *Desulfovibrio gigas* hydrogenase) (16). Modewise, these modifications would have evolved to increase the availability of electrons in the relay, for rapid transfer back to the active site when O$_2$ is present and enters the buried active site, a package of electrons derived from the respiratory-chain quinones. If oxygen is present and enters the buried active site, a package of electrons, derived from hydrogen oxidation and held in the iron-sulfur cluster relay system, transfers back to convert it cleanly to water. The enzyme thus avoids production of reactive oxygen intermediates that would otherwise cause inactivation. This study establishes the basis of the oxygen tolerance in respiratory nickel-iron hydrogenases and demonstrates biology’s unexpected use of nickel in the active site of a four-electron oxidase.

Significance

Mass spectrometry experiments with a nickel-containing respiratory hydrogenase from *Escherichia coli* provide conclusive proof that it catalyzes the four-electron reduction of oxygen by hydrogen, a reaction analogous to combustion. Hydrogenase-1 is a membrane-bound enzyme that oxidizes hydrogen in the periplasm to reduce respiratory-chain quinones. If oxygen is present and enters the buried active site, a package of electrons, derived from hydrogen oxidation and held in the iron-sulfur cluster relay system, transfers back to convert it cleanly to water. The enzyme thus avoids production of reactive oxygen intermediates that would otherwise cause inactivation. This study establishes the basis of the oxygen tolerance in respiratory nickel-iron hydrogenases and demonstrates biology’s unexpected use of nickel in the active site of a four-electron oxidase.

Author contributions: P.W., C.C.D., and F.A.A. designed research; P.W. and C.C.D. performed research; P.W., C.C.D., and F.A.A. analyzed data; and P.W., C.C.D., F.S., and F.A.A. wrote the paper.

The authors declare no conflict of interest.

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6606–6611  |  PNAS  |  May 6, 2014  |  vol. 111  |  no. 18  
www.pnas.org/cgi/doi/10.1073/pnas.1322393111
attacks. Combined with the minimal Ni(II) to Ni(III)-OH conversion as Ni-B is formed, all four electrons needed for complete O2 reduction are poised for transfer (9).

We now prove that Hyd-1 from *E. coli* is a four-electron H2/O2 oxidoreductase by analyzing the different oxygen products formed during long-term steady-state catalytic oxidation of H2 by O2 (these being, respectively, the sole electron donor and acceptor). Hyd-1 and other enzymes of this class are thus unique in two respects: first, in catalyzing a classic combustion reaction, the reaction of H2 with O2; and second, in using Ni to achieve this activity. Before completion of this manuscript, a paper describing the O2 inventory for a soluble cytoplasmic flavohydrogenase from *Ralstonia eutropha* was published, which showed that H2O is produced from both flavin and hydrogenase subunits, along with equivalent amounts of H2O2 (17). The studies we now describe, on a structurally characterized periplasmic hydrogenase that experiences substantial environmental O2 levels in vivo, unambiguously establish the 4 e− oxidase activity of a Ni-containing center, placing Ni among Cu and Fe in mediating this reaction in biology. We are able to correlate the H2O formation rates by *E. coli* Hyd-1 with complementary O2 tolerance data from recent electrochemical studies on the same enzyme and find good agreement.

**Results**

To determine the products and intermediates produced when Hyd-1 reacts exclusively with H2 and O2 in aqueous solution, we measured the time courses for production of H2O, O2− and H2O2 during prolonged exposure to a H2/O2 gas mixture. The H218O produced under H2/18O2 mixtures was determined by isotope ratio mass spectrometry (IRMS) after equilibrating sampled aliquots with CO2 (Methods). Formation and decay of reactive oxygen species O2− and H2O2 were measured colorimetrically.

**Determination of Water Production by Mass Spectrometry.** Incubation of Hyd-1 in aqueous buffer under 90% H2 and 10% 18O2 yielded large amounts of H218O. Fig. 3 A shows that the asterisk indicates the labeled 18O introduced and measured in our experiments.

**Fig. 1.** Simplified catalytic cycles of Hyd-1. Hydrogen oxidation (Left) proceeds at rates exceeding 100 s−1 and feeds electrons into the relay system of FeS clusters. Oxygen reduction to water (Right), which begins with O2 attack on the active site to form Ni-B (kI), consumes the electrons stored in the FeS relay. Reactivation of Ni-B (kA), to reenter either catalytic cycle, is fast and involves reduction by one electron, most likely producing the species known as Ni-SI (9). The stoichiometries are displayed in accordance with spectroscopic results showing that the OH bridging ligand in the Ni-B state originates from solvent, not from O2 (45, 46). The asterisk indicates the labeled 18O introduced and measured in our experiments.

**Fig. 2.** (A) *E. coli* hydrogenase-1 is a (αβ)2 homologous heterodimer (functional monomers are shown in gray and green) consisting of large α subunits (light gray and light green) containing the active site and small β subunits (dark gray and dark green) containing the FeS cluster relay. The cytochrome subunit (red) contains a b-type heme group (Protein Data Bank, PDB: 4GD3) (11). Likely sites of reaction with O2 are shown alongside putative products. The crystal structure suggests that in vivo there should be two cytochromes attached and indicates that, whereas the remaining cytochrome might be stable in solution, the heme groups are not. (B) Proximal clusters. (Upper) A conventional [4Fe-4S] cluster, most commonly ligated by four cysteines, is normally noted for its property of undergoing a fast, one-electron transfer reaction using the [4Fe-4S]2+/1+ couple. Further oxidation to the 3+ level (“super oxidation,” Super Ox) is irreversible (structure of cluster is that found in the [NiFe]-hydrogenase from *Desulfovibrio fructosovorans*, PDB: 1FRF). (Lower) The special [4Fe-3S] cluster, found in membrane-bound O2-tolerant hydrogenases, is ligated by six cysteines and is able to undergo two rapid and sequential one-electron transfers involving the couples [4Fe-3S]4+/3+ and [4Fe-3S]5+/4+. Super oxidation to give the 5+ species (Super Ox) is fully reversible and is a proton-coupled electron-transfer process that results in formation of a Fe-N(peptide) bond (structure of cluster is that found in *E. coli* Hyd-1 reduced and super oxidized forms; PDB: 3UQY and 3USC, respectively) (10). Figures were created using PyMOL.

Wulff et al. PNAS | May 6, 2014 | vol. 111 | no. 18 | 6607
accumulated H₂¹⁸O increases robustly with time from approximately 1.148 ± 642 μM/μM Hyd-1 after 30 min to 5,003 ± 1,911 μM/μM Hyd-1 after 120 min. The rate of sustained reductive formation of H₂¹⁸O from H₂¹⁸O₂ by Hyd-1 was estimated from measurements made over a wide range of incubation times (Fig. 4). A straight line with a fixed intercept on the origin was fitted to the data for the native enzyme, yielding a slope corresponding to a H₂¹⁸O turnover rate of approximately 0.65 μM₁₈H₂O/μMHyd-1 s⁻¹. The scatter is attributed to difficulties in delicate sample handling before mass spectrometry; notably, the SDs for the mass spectrometry measurements for each experiment lay within the range of 4.8–17.8 μM H₂¹⁸O. The rate of H₂¹⁸O₂ formation under 10% ¹⁸O₂ is at least twice that under 5% ¹⁸O₂ (based on limited data for 5% O₂ after 3 and 4 h) (Fig. 4). Therefore, 10% O₂ does not cause inactivation over a period of at least 4 h and the enzyme activity is maintained at a constant level.

A variant with a substitution in the small subunit (P242C) was studied for comparison with native enzyme. This variant, in which the medial [3Fe-4S] cluster is converted to a [4Fe-4S] cluster (18), retains high rates of H₂ oxidation and resistance to short-term O₂ exposure but has severely impaired long-term O₂ tolerance (9). The P242C variant shows much decreased H₂¹⁸O production and essentially no further increase beyond 120 min.

**Formation of Hydrogen Peroxide.** Hydrogen peroxide was analyzed using 10-acetyl-3,7-dihydroxyphenoxazine (Ampliflu Red), which in the presence of H₂O₂ is irreversibly converted to the fluorescent dye 7-hydroxy-3H-phenoxazin-3-one (Resorufin) by horseradish peroxidase. This assay was used previously to monitor H₂O₂-evolving side reactions simultaneously with the main redox reaction of an enzyme (19). However, in the presence of H₂O₂, Hyd-1 reduces Resorufin (a quinone) and the product reacts with O₂ to give reactive oxygen species, including H₂O₂ (20). It was therefore prudent to take endpoint, rather than concurrent, H₂O₂ measurements after stopping the reaction by purging the sample solution with Ar. Incubation of Hyd-1 in 90% H₂ and 10% O₂ yielded low levels of H₂O₂ that appeared independent of reaction time—a typical result being 22 μM H₂O₂ in the presence of 0.15 μM Hyd-1. After 30 min, the H₂O₂/Hyd-1 ratio in solution was 135.7 ± 10.9 μM H₂O₂/μM Hyd-1 (Fig. 3A), whereas after 60 and 120 min, the corresponding values were: 137.7 ± 7.6 μM and 158.4 ± 32.5 μM. In the absence of O₂, no H₂O₂ was found.

**Decomposition of Hydrogen Peroxide.** Voltammetric measurements of catalytic H₂ oxidation showed that Hyd-1 is stable in the presence of 1 mM H₂O₂ for at least several hours. Provided H₂ was bubbled into the cell solution (which efficiently removes traces of O₂ formed by peroxide decomposition) the catalytic cyclic voltammograms (+0.3 V to −0.3 V) were identical to those measured in the absence of H₂O₂ (5, 7). Incubation of Hyd-1 with known starting concentrations of H₂O₂ under 1 atm H₂ and no O₂ showed that the H₂O₂ concentration decreased with time (Fig. 3B, Left). Some of the decrease could be attributed to nonenzymatic activity that was minimized by sealing the clean reaction vessel under N₂ flow. Importantly, the removal of

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**Fig. 3.** (A) O₂-reduction products. Concentrations (product per enzyme) of O₂⁻, H₂O₂, and H₂¹⁸O, after 30, 60, and 120 min under 90% H₂ and 10% ¹⁸O₂ at pH 7.0, 20 °C. Error bars represent SD (n = 3). (B) Peroxidase activity. (Left) Decrease in H₂O₂ concentration from 20 and 40 μM starting solutions after 30 and 60 min with 0.15 μM Hyd-1 under 1 atm H₂ at pH 7.0, 20 °C, including background decomposition. (Right) Decrease in H₂O₂ concentration from 27 μM H₂O₂ starting solution after 30 min with 0.225 μM Hyd-1 under 1 atm N₂ or H₂ at pH 7.0, 20 °C excluding background decomposition. Error bars represent SD (n = 3).

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**Fig. 4.** Determination of the rate of H₂O formation by Hyd-1. The micromole-level concentrations of H₂¹⁸O in 1-mL reaction samples were determined by mass spectrometry and divided by the micromole concentration of enzyme used. Native enzyme (empty squares) and P242C variant (black diamonds) were measured after incubation in 90% H₂ and 10% ¹⁸O₂ atmosphere at pH 7 and 20 °C. Further native samples (black-and-white squares) were also measured in the same buffer but under only 5% ¹⁸O₂ and 95% H₂ for comparison. The slope of the extrapolated fit (dashed line) to the 10% ¹⁸O₂ native data points (empty squares) yields an approximate H₂¹⁸O formation rate of 0.65 s⁻¹.
H$_2$O$_2$ that depended on Hyd-1 depended also on the presence of H$_2$ (Fig. 3B, Right). The apparent rate constant for the decrease in H$_2$O$_2$ concentration (which is therefore due to a peroxidase-like activity) was calculated by dividing the decreases after 30 min by both time and initial H$_2$O$_2$ concentration, i.e., 20 and 40 $\mu$M (Fig. 3B, Left). An average value of 3.2 $\pm$ 0.1 x $10^{-5}$ $\mu$M$_{H_2O_2}^{-1}$ s$^{-1}$, including background contributions, was obtained with 0.15 $\mu$M Hyd-1.

The fact that H$_2$O$_2$ levels remain constant over time (Fig. 3A) in the presence of H$_2$ and O$_2$ but decrease when O$_2$ is absent (Fig. 3B) shows that H$_2$O$_2$ is maintained at a steady state. For a steady-state concentration of approximately 22 $\mu$M H$_2$O$_2$ (Fig. 3A) and 0.15 $\mu$M Hyd-1, as in the above experiment, a total H$_2$O$_2$ decomposition rate of 0.007 s$^{-1}$ (from 3.2 $\times$ 10$^{-5}$ $\mu$M$_{H_2O_2}^{-1}$ s$^{-1}$ $\times$ 22 $\mu$M$_{H_2O_2}$) is estimated, which must equal the H$_2$O$_2$ evolution rate. Therefore, the rate constant for peroxide formation $k_{cat}^{\text{NO}_2}$ = 0.007 s$^{-1}$/0.15 $\mu$M$_{Hyd-1}$ = 0.047 $\mu$M$_{H_2O_2}$ $\mu$M$_{Hyd-1}$ s$^{-1}$.

**Detection of Superoxide.** Superoxide produced throughout the reaction of Hyd-1 with H$_2$O$_2$ was assayed using the reaction with hydroxylamine (21), yielding NO$_2^-$ that was detected with Griess reagent. Tests showed that hydroxylamine does not inhibit Hyd-1 activity (22) or react with H$_2$O$_2$ to form nitrite (23). In reactions of Hyd-1 with 90% H$_2$ and 10% O$_2$ (Fig. 3A) the amount of NO$_2^-$ scaled with time (5.8 $\pm$ 0.9 $\mu$M to 18.7 $\pm$ 1.6 $\mu$M $\mu$M$_{Hyd-1}$ over 30–120 min) and linearly with enzyme concentration (after 30 min with 0.15 $\mu$M or 0.45 $\mu$M Hyd-1 $\sim$ 7.6 $\pm$ 1.1 $\mu$M and 7.4 $\pm$ 2.1 $\mu$M NO$_2^-$, respectively, were determined per micromole of Hyd-1). In view of the disagreement in the literature regarding the stoichiometry of the hydroxylamine/O$_2$/$\text{NO}_2^-$ assay, we adopted the stoichiometric ratio of 1.3 O$_2$/$\text{NO}_2^-$ rather than 2.0 as proposed by Elstner and Heupel (21) and Kono (24) or 1.0 as used by Schneider and Schlegel (22). Unlike H$_2$O$_2$, the amount of NO$_2^-$ increased linearly with time and a rate constant $k_{cat}^{\text{NO}_2^-}$ of 3.2 $\pm$ 0.4 $\times$ 10$^{-3}$ s$^{-1}$ was calculated, giving a superoxide production rate of 4.1 $\pm$ 0.6 $\times$ 10$^{-3}$ $\mu$M$_{O_2}$ $\mu$M$_{Hyd-1}$ s$^{-1}$.

To estimate how much H$_2$O$_2$ results from superoxide, we measured H$_2$O$_2$ equilibrium levels in the presence and absence of hydroxylamine. Incubation of 0.225 $\mu$M Hyd-1 under 90% H$_2$ and 10% O$_2$ at 20 °C and pH 7 yielded 18.5 $\pm$ 1.2 $\mu$M and 26.4 $\pm$ 1.6 $\mu$M H$_2$O$_2$, respectively, suggesting a 30% decrease in O$_2$ availability, for H$_2$O$_2$ formation, due to scavenging by hydroxylamine.

**Assay of Intermolecular Electron-Transfer Kinetics Using Cytochrome c.** The partially acetylated form of cytochrome c (horse heart), which is often used in superoxide assays to minimize interactions with enzymes, was rapidly reduced by Hyd-1 in H$_2$-saturated buffer, even in the absence of O$_2$ (thus precluding its use for superoxide analysis). An apparent rate constant of ~53 s$^{-1}$ (mols cytochrome c per mole Hyd-1 for electron transfer from Hyd-1 to cytochrome c was calculated, compared with 58 s$^{-1}$ for nonacetylated cytochrome c. These measurements reaffirmed the high activity of Hyd-1 for discharging electrons derived from H$_2$ over a long range to both electrodes and large macromolecules.

**Assay of Hydrogen Oxidation Activity.** The H$_2$ oxidation activity of Hyd-1 was assayed in solution using benzyl viologen (BV), as described previously (3). In H$_2$-saturated buffer, an initial turnover frequency for H$_2$ oxidation ($k_{cat}^{H_2}$) of approximately 125 H$_2$ s$^{-1}$ was observed at pH 7.0, 20 °C. Owing to the marginal driving force provided by oxidized benzyl viologen, this value is likely to be a significant underestimate of the true H$_2$oxidation activity.

**Discussion**

A model proposed to account for the O$_2$ tolerance of respiratory [NiFe]-hydrogenases is based on the ability of the enzyme to supply, very rapidly, four electrons (and protons) to the active site when O$_2$ attacks. In such a way, the effect of O$_2$ is merely to suppress H$_2$ oxidation activity, and potentially damaging, trapped intermediate oxygen species are bypassed. Were this direct four-electron reduction to be the sole fate of all of the O$_2$ molecules encountering Hyd-1 in the presence of H$_2$, no H$_2$O$_2$ or O$_2^-$ would be detected; however, this ideal situation is wholly unrealistic. The high rate at which Hyd-1 catalyzes cytochrome c reduction by H$_2$ shows immediately that Hyd-1 under H$_2$ also provides a proficient source of electrons for remote outer-sphere 1-e$^-$ reactions.

**Direct Reduction of Oxygen to Water.** At 0.65 $\mu$M H$_2$O$_2$ $\mu$M$_{Hyd-1}$ $^{-1}$ s$^{-1}$, the rate of formation of H$_2$O$_2$, approximately constant throughout 4 h of reaction, corresponds to a 18O$_2$ removal rate of 0.325 $\mu$M$_{O_2}$ $\mu$M$_{Hyd-1}$ $^{-1}$ s$^{-1}$ under 10% O$_2$. In contrast, the H$_2$O$_2$ concentration soon attains a low steady-state level, meaning that its rate of production from O$_2$ becomes equal to its rate of disappearance, i.e., formation of 0.047 $\mu$M$_{H_2O_2}$ $\mu$M$_{Hyd-1}$ $^{-1}$ s$^{-1}$. Thus, regardless of how H$_2$O$_2$ is generated, its maximum rate of formation is six to seven times lower than the rate at which H$_2$O$_2$ is formed from 18O$_2$, confirming that a direct 4e$^-$ reduction is the dominant pathway. For simplicity, we henceforth express all rate constants that depend directly on enzyme concentration as turnover frequencies, i.e., mol substrate/mol enzyme with units of s$^{-1}$.

Correcting for the production of H$_2$ as $\text{H}_2$O$_2$ via Hyd-1, the rate constant for direct reduction of O$_2$ to H$_2$O is therefore 0.325–0.047 $= 0.28$ s$^{-1}$, accounting for 86% of the O$_2$ consumed. Because direct reduction of O$_2$ to H$_2$O requires a special site with the ability to provide all four electrons (and protons), and free peroxide is reduced much more slowly, we conclude that 86% of the O$_2$ is reduced to H$_2$O by direct reaction at the [NiFe] site.

**Indirect Reduction of Oxygen to Water.** The fraction of H$_2$O$_2$ identified as originating from O$_2$ $^*$ using the hydroxylamine assay was between 9 and 30%. The mechanism and efficiency of the hydroxylamine assay being rather poorly understood (25), this fraction might indeed be higher. Disproportionation of O$_2$ into H$_2$O$_2$ in water at pH 7 is sufficiently fast ($6 \times 10^{-3}$ M$^{-1}$ s$^{-1}$) (26) that at micromolar concentrations most of the H$_2$O$_2$ could originate from O$_2$ if the latter is not intercepted by hydroxylamine. Referring to Fig. 2A, formation of H$_2$O$_2$ from O$_2$ without disproportionation of O$_2$ requires either that: (i) O$_2$ reacts at a site with an inherent two-electron capability—candidates being the [NiFe] active site (in which case peroxide is obviously released harmlessly), a site derived from the heme-containing subunit, or the proximal [4Fe-3S] cluster, or (ii) O$_2$ undergoes two rapid consecutive one-electron reactions in which the superoxide intermediate is reduced further before it can escape. A possible location for the latter reaction is between the two adjacent distal clusters. Production of reactive oxygen species is unavoidable in enzymes that have accessible, low-potential centers, such as mitochondrial complexes I and II (27, 28). The slow peroxidase activity of Hyd-1 probably stems from action of the labile heme-b component with axial ligand disruption, not unlike the microperoxidase activity of cytochrome c samples (29). The purified E. coli hydrogenase-1 stock solutions used in these experiments were examined by UV-visible spectrophotometry to establish the content of associated and/or copurified cytochrome b (11). The ratio of b-type heme to Hyd-1 dimer varied between 0.41 and 0.47.

**Comparison of Oxygen Reduction Rates with the Model and Electrochemical Data.** The rate constant (0.28 s$^{-1}$) for direct conversion of 18O$_2$ to H$_2$O$_2$ is fully consistent with that expected from the model and electrochemical experiments reported...
Oxidase Activity and Its Physiological Role. An implicit prediction of the model is that O2-tolerant respiratory [NiFe]-hydrogenases are high-fidelity four-electron oxidases (Fig. 1), thus extending the substrate range that includes cytochrome c oxidase (Fe,Cu) (30), blue copper oxidases (Cu) (31), and alternative oxidases (Fe) (32) to include the element Ni as an active component. Compared with the established enzymes, the special oxidase activity of Hyd-1 is low, even under artifically high O2 concentrations, consistent with it serving to protect the active site, rather than sequestering O2 from the periplasm.

In *E. coli*, Hyd-1 is anchored to the periplasmic side of the inner membrane where its role is to pass electrons from H2 oxidation through its cytochrome b partner to the cytoplasmic side of the membrane where ubiquinone is reduced to ubiquinol (33–35). The enzyme is synthesized under anaerobic conditions, and its expression is switched off in a fully oxic environment (36–38). A coexpressed cytochrome bd-II oxidase (39) is thought to complete this short respiratory electron transport chain between H2 and O2 under microaerobic conditions. In the case of O2 attack at the Hyd-1 active site, the electron flow is temporarily reversed within the (op)2 dimer as complete reduction of O2 takes place. Thus, we observe a short circuit, burning off O2 as a rescue mechanism.

Avoiding damage to the active site through the four-electron reduction activity that involves rapid formation of Ni-B, a well-characterized Ni(III)-OH species, is energetically essential for the organism. The native enzyme tolerates high O2 concentrations (10%) indefinitely (Fig. 4); indeed ~10,000 turnovers (O2 to 2 H2O) have occurred after 4 h without the rate decreasing. In contrast, Hyd-1 proximal and distal cluster variants (including P242C) are unable to neutralize O2 with any fidelity and the enzyme soon becomes inactive (9, 14).

A Role for Nickel in Early Adaptation to Oxygen. Nickel, an element not normally associated with enzymatic O2 activation, was available to biology much earlier in Earth’s history than copper (40, 41); in fact [NiFe]-hydrogenases are considered to be among the most ancient enzymes (42). Hydrogen sourced from geological processes (e.g., hydrothermal circulation through basalt and serpentine, arc volcanism, and ridge-axis volcanism) was available to metabolism long before atmospheric levels of O2 began to rise (41). A slow rise in O2 concentrations would have driven hydrogen-ase evolution, first to merely survive (transient) O2 exposure, and later to develop true O2 tolerance to sustain H2 oxidation in a microaerobic respiratory chain with newly evolved full-fledged terminal, iron-containing cytochrome bd oxidases.

Methods

Hyd-1 was purified as described previously [native (3), P242C (15)]. Stock solution concentrations were determined with Bradford reagent (Sigma; B6916). The cytochrome b/hydrogenase ratio was evaluated from the absorption spectra of H2-reduced and air-oxidized samples using a home-built extinction coefficient $e_{\text{absorb}}$ of 22 mm$^{-1}$ cm$^{-1}$ (43) and molecular masses of 27.6 kDa, 36.8 kDa, and 64.6 kDa for cytochrome b, small and large Hyd-1 subunits, respectively (11). Preparation and handling of Hyd-1 samples were carried out in N2-filled glove boxes. Glassware was cleaned, either by simply rinsing with ultrapure water (Milli-Q) or by rinsing with acetone, ethanol, ultrapure water and ethanol, with subsequent drying and sealing under a flow of pure nitrogen to avoid contamination with airborne impurities.

Reactions of Hyd-1 with H2 and 18O2 were carried out in glass vials with septa-covered inlets and a three-way tap for selective addition of sample and gases. Solutions of Hyd-1 (0.1–1.0 μM) were incubated in 1 mL mixed buffer solution (0.1 M NaCl, 15 mM Mes (N-tris(hydroxymethyl)methyl)-3-aminopropanesulfonic acid), 15 mM CHAPS (3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonate), 15 mM sodium acetate; all Sigma) at pH 7.0, 20°C for the indicated time periods after flushing the reaction chamber with H2 and introducing the appropriate amount of 18O2 (99.9%; Cambridge Isotope) gas via the three-way tap and a syringe. The solution was stirred throughout. The final volume was 6 mL (solution plus headspace). After the reaction, the samples were stored at 4°C in glass vials with microaerobic headspace before performing mass spectrometry.

Oxygen isotope measurements were performed with a Delta V Advantage isotope mass spectrometer fitted with a Gas Bench II device, using the method described by Nelson (44). Iso-analytical limited standards IA-ROS2 and IA-ROSS were used for two-point linear normalization with IA-ROSS = 19.64 ± 0.11% and IA-ROS2 = 108.63 ± 0.33% relative to Vienna Standard Mean Ocean Water (VSMOW). 2 External error was calculated and checked from repeat measurements of iso-analytical standard IA-ROS4, with two-point normalized results ($\delta^{18}O_{\text{VSMOW}} = 0.53 ± 0.11$, n = 21) within the error of iso-analytical limited laboratory results ($\delta^{18}O_{\text{IA-ROS4}} = 0.36 ± 0.23$, n = 20). Results are expressed on the same normalized scale such that $\delta^{18}O$ of Standard Light Antarctic Precipitation 2 reference water is −55.5%. Corrected $\delta^{18}O_{\text{VSMOW}}$ vs. VSMOW values were converted to micromole concentration increases relative to enzyme-free samples (blank reactions) assuming a concentration of 55.5 M H2O in aqueous buffer and an isotopic ratio $^{18}O^{16}O$ vs. VSMOW of 2.05 ppm: $\delta^{18}O$ increase (i.e., deviation of the $^{18}O^{16}O$ ratio for the sample from the reference value) of 1‰ = 2 ppm = 111 μM H2O. Results were evaluated by comparison with O2-exposed (unlabeled) control reactions.

Hydrogen peroxide was assayed under the same buffer conditions as for the $^{18}O$ experiments. After incubation in H2/O2 (BOC Industrial Gases) gas mixtures for the specified times, the reaction solution was purged with argon and three volumes of assay solution, containing 100 μM 10-acetyl-3,7-dihydroxyphenoxazin (Ampilflu Red) and 2.2 units/mL horseradish peroxidase (all from Sigma), were added. After 15 min, the absorbance was measured at 571 nm (formation of Resorufin). Calibrations were carried out with H2O2 standards in the range of 2–40 μM.

To estimate superoxide, 1 mM hydroxylamine (Sigma) was added to the reaction buffer described above. Hydroxylamine reacts rapidly with superoxide, resulting in formation of nitrite. After flushing with argon at the end of the reaction, 300 μL sample was added to 300 μL of assay solution (modified Griess reagent; Sigma) and the absorbance was recorded at 540 nm. The assay was calibrated against known NaN3 (Fisher) concentrations.

Hydrogen oxidation activity was assessed using benzyl viologen in H2-saturated buffer (50 mM Tris-HCl, 100 mM NaCl, 25 mM benzyl viologen, pH 7) at 20°C (all from Sigma). Initial absorbance increase rates were converted into catalytic rates using an absorbance coefficient for BV of $e_{\text{absorb}} = 9.82$ mm$^{-1}$ cm$^{-1}$. Rates of cytochrome c reduction in solution were determined by recording the increase in absorbance at 550 nm. Hydrogen-saturated solutions of cytochrome c and partially acetylated cytochrome c (from equine heart, $e_{\text{absorb}} = 29.5$ and 23.5 mm$^{-1}$ cm$^{-1}$; Sigma) at 0.6 mg/mL and 0.8 mg/mL, respectively, were used with 0.12 μM Hyd-1 in mixed buffer (see above). Protein film electrochemistry experiments, carried out as described previously, were used to establish the stability of Hyd-1 in the presence of H2O2 (9).

Acknowledgments. We thank Gideon Henderson for discussion of the isotope experiments. Research was supported by the Biological and Biotechnological Sciences Research Council (Grants BB/H036373/1 and BB/K02086X/1 to F.A.A. and BB/H001190/1 and BB/02008X/1 to F.A.A. and BB/H001190/1 to F.A.A. and BB/02008X/1 to F.S.) and St John’s College, Oxford through award of a graduate scholarship (to P.W.). F.A.A. is a Royal Society–Wolfson Research Merit Award holder.


