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The [NiFe]-Hydrogenase of *Pyrococcus furiosus* Exhibits a New Type of Oxygen Tolerance

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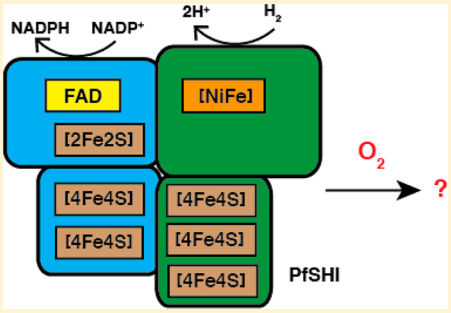
Supporting Information

ABSTRACT: We report the first direct electrochemical characterization of the impact of oxygen on the hydrogen oxidation activity of an oxygen-tolerant, group 3, soluble [NiFe]-hydrogenase: hydrogenase I from *Pyrococcus furiosus* (*Pf*SHI), which grows optimally near 100 °C. Chronoamperometric experiments were used to probe the sensitivity of *Pf*SHI hydrogen oxidation activity to both brief and prolonged exposure to oxygen. For experiments between 15 and 80 °C, following short (<200 s) exposure to 14 μM O_2 under oxidizing conditions, *Pf*SHI always maintains some fraction of its initial hydrogen oxidation activity; i.e., it is oxygen-tolerant. Reactivation experiments show that two inactive states are formed by interaction with oxygen and both can be quickly (<150 s) reactivated. Analogous experiments, in which the interval of oxygen exposure is extended to 900 s, reveal that the response is highly temperature-dependent. At 25 °C, under sustained 1% O_2/ 99% H_2 exposure, the H_2oxidation activity drops nearly to zero. However, at 80 °C, up to 32% of the enzyme's oxidation activity is retained. Reactivation of *Pf*SHI following sustained exposure to oxygen occurs on a much longer time scale (tens of minutes), suggesting that a third inactive species predominates under these conditions. These results stand in contrast to the properties of oxygen-tolerant, group 1 [NiFe]-hydrogenases, which form a single state upon reaction with oxygen, and we propose that this new type of hydrogenase should be referred to as oxygen-resilient. Furthermore, *Pf*SHI, like other group 3 [NiFe]-hydrogenases, does not possess the proximal [4Fe3S] cluster associated with the oxygen tolerance of some group 1 enzymes. Thus, a new mechanism is necessary to explain the observed oxygen tolerance in soluble, group 3 [NiFe]-hydrogenases, and we present a model integrating both electrochemical and spectroscopic results to define the relationships of these inactive states.



INTRODUCTION

Hydrogenases, the enzymes that catalyze H^+/H_2 interconversion, have received widespread attention as models for multielectron redox catalysis at nonprecious metal ([FeFe]- or [NiFe]-) active sites.[1−7] Although the high catalytic rates of these enzymes make them technologically appealing,[8−10] the active sites are usually extremely sensitive to redox potential as well as small molecule inhibitors such as oxygen and carbon monoxide, limiting their technological utility.[11−13] In this respect, they are similar to many other energetically important redox metalloenzymes including nitrogenases and some formate dehydrogenases.[14−19]

Compared to the irreversible inactivation of many metalloenzymes, particularly [FeFe]-hydrogenases, by oxygen, [NiFe]-hydrogenases are less sensitive to oxygen since they are reversibly inactivated.[11,20−22] [NiFe]-hydrogenases are often divided into two categories based on reactivity with oxygen: standard and oxygen-tolerant.[23] Standard [NiFe]-hydrogenases, such as those from *Escherichia coli* (Hyd2), *Desulfovibrio fructosovorans* (*Df*Hase), and the *Allochromatium vinosum* membrane-bound enzyme, are reversibly inactivated by exposure to oxygen to form a mixture of two distinct, inactive states referred to as "ready" and "unready", or Ni-B and Ni-A, respectively. Reactivation of Ni-B occurs on a time scale of seconds, whereas reactivation of Ni-A requires more prolonged exposure to reductive conditions.[11,24−30] A second group, called "oxygen-tolerant" [NiFe]-hydrogenases, has been identified based on their ability to maintain some hydrogen oxidation activity for extended periods in the presence of oxygen. This group includes such enzymes as the membrane-bound hydrogenases (MBHs) from *Ralstonia eutropha* (*Re*MBH),[20,31,32] *Aquifex aeolicus*,[33,34] *Escherichia coli* (*Ec*Hyd-1),[35,36] and *Hydrogenovibrio marinus*.[37,38] These hydrogenases are believed to resist inactivation by oxygen by forming only the Ni-B state of the active site in the presence of oxygen and by having a relatively high reduction potential for reactivation. This resistance to formation of Ni-A has been hypothesized to arise from the ability to reduce attacking oxygen completely with four electrons and prevent formation of any partially reduced blocking species.[27,28,33,36] In support of this hypothesis,

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*Re*MBH and *Ec*Hyd1 have been shown to be able to reduce oxygen to form water.[39,40]

Early research suggested that oxygen tolerance in the standard [NiFe]-hydrogenases might arise from restricted access of oxygen to the active site,[41−45] but more recent evidence has led to the hypothesis that interactions between the active site and the adjacent [FeS] clusters that provide reducing equivalents from external partners are the main factors controlling oxygen tolerance. X-ray crystal structures determined from three oxygen-tolerant MBHs from different biological sources revealed a [4Fe3S] cluster proximal to the hydrogen-activating site. This cluster is thought to be stable in the 3+, 2+ and 1+ oxidation states under physiological conditions.[34,37,46−52] This capacity to take up two electrons may be key to quickly and simultaneously providing multiple electrons to the active site to reduce oxygen and prevent formation of the Ni-A "unready" state. Lending further support to this hypothesis, the unusual cluster is coordinated by a six cysteine-containing primary sequence motif that is highly conserved among oxygen-tolerant MBHs.[47,53,54] Similarly, based on the decreased oxygen tolerance of site-directed mutants, the medial [3Fe4S] cluster of *Ec*Hyd1 has also been hypothesized to play a role in oxygen tolerance.[55] This may be explained by the relatively high reduction potential of the $[3Fe4S]^{+/0}$ couple which leaves this cluster reduced, i.e., with an electron available to donate to the active site, under most physiologically relevant conditions. Finally, replacing a non-metal coordinating valine (74), located between the active site and the proximal cluster, with a cysteine in the *Df*Hase enzyme created a more oxygen-tolerant enzyme without affecting the reduction potentials of the proximal cluster.[56,57] Recent X-ray crystal structures of this variant enzyme suggest that the point mutation causes a change in the first coordination sphere of the Ni to include a mainchain carboxamido nitrogen.[58] This suggests that the protein matrix between the active site and the proximal cluster also plays a crucial role in oxygen tolerance, perhaps by modulating electronic coupling between the cofactors or influencing active site structure and dynamics.

[NiFe]-hydrogenases are phylogenetically divided into four diverse groups, but the majority of mechanistic studies have utilized enzymes from group 1, which are mainly membrane-bound enzymes physiologically associated with hydrogen oxidation to provide reducing equivalents directly to respiratory systems.[59] On the other hand, group 3 [NiFe]-hydrogenases are soluble enzymes that couple oxidation of hydrogen to reduction of cytoplasmic redox cofactors such as cofactor F_{420} and the pyridine nucleotides NAD^+ or $NADP^+$.[60] Unlike group 1 hydrogenases, which are usually isolated as heterodimers consisting of the large hydrogen-activating subunit and the small [FeS]-containing subunit, group 3 soluble hydrogenases (SHs) contain not only the dimeric hydrogenase component but also a multimeric, flavin-containing diaphorase. Previous studies have shown that the SHs of *R. eutropha* (*Re*SH) and *Pyrococcus furiosus* (*Pf*SHI) are, to some extent, oxygen-tolerant; i.e., they maintain activity for extended periods in solution assays in the presence of oxygen.[61−63] However, these enzymes do not possess the additional cysteine residues near the proximal cluster that have come to be the hallmark of oxygen-tolerant group 1 [NiFe]-hydrogenases. This makes them unlikely to have a modified proximal [FeS] cluster and raises the question of how oxygen tolerance is achieved in the group 3 SHs. Lenz and co-workers have suggested that the unusual spectroscopic properties (FTIR) of the [NiFe]-site of *Re*SH can be explained by reversible sulfoxygenation of active site cysteines and that this reaction may play a role in oxygen tolerance, although there is no evidence to support this.[64]

Protein film electrochemistry (PFE), a technique in which a redox protein is functionally adsorbed to an electrode and electron transfer reactions can be observed without a chemical mediator,[65] has proven invaluable for characterizing the differences in the reactivity of oxygen-sensitive and oxygen-tolerant group 1 [NiFe]-hydrogenases.[11,13,66−69] The main advantage of PFE over solution assays is that the need for mediators, redox active molecules that themselves usually react with oxygen, can be eliminated, and the catalytic activity at precisely defined electrochemical potential and solution conditions can be directly monitored as current. In spite of these advantages, only two SHs have been characterized using PFE: the dimeric hydrogenase subcomplex of *Re*SH and the complete tetrameric holoenzyme from *Synechocystis* sp. PCC6803 (*Syn*SH).[62,70] Both of these enzymes proved to be oxygen-sensitive. Thus, the reactions of an oxygen-tolerant SH have not yet been characterized using this technique.

Herein, we present the first investigation via PFE of the reactions of oxygen with an oxygen-tolerant, soluble, group 3 [NiFe]-hydrogenase, *Pf*SHI. The hyperthermophile *P. furiosus* grows at temperatures near 100 °C in its native environment, and, as such, the electrocatalytic characteristics of *Pf*SHI have been investigated at both high and low temperatures. Experiments wherein the enzyme is subjected to both short- and long-term O_2 exposure have also been conducted to study the kinetics of inactivation and reactivation in the enzyme. We show that, unlike oxygen-tolerant, group 1 [NiFe]-hydrogenases that inactivate to form a single state, following transient exposure to oxygen, *Pf*SHI reacts with oxygen to form two distinct states that are distinguished by the electrochemical potential required to reactivate them. Furthermore, prolonged exposure to oxygen, especially at high temperature, forms an additional, slowly reactivating state not previously described. This is the first demonstration that oxygen tolerance and formation of a single inactive state are not mutually inclusive. The properties of *Pf*SHI are compared to both oxygen-tolerant and oxygen-sensitive group 1 [NiFe]-hydrogenases as well as *Syn*SH, and possible mechanisms for oxygen tolerance in SHs are considered.

MATERIALS AND METHODS

The Strep- and polyhistidine-tagged forms of *Pf*SHI were over-produced in *P. furiosus* and purified as described by Chandrayan et al.[71,72] Protein film electrochemistry (PFE) was performed in an anaerobic glovebox as described by McIntosh et al.[70] All chemicals were of the highest grade commercially available and were used without further purification. Solutions for electrochemical experiments were prepared using purified water (resistivity 18.2 MΩ cm^{-1}). The mixed buffer consisted of 15 mM each of 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), 2-[*N*′-cyclohexyl-amino]-ethane-sulfonic acid (CHES), 2-[*N*′-morpholino]ethane-sulfonic acid (MES), *N*′-tris[hydroxymethyl]methyl-3-amino-propane-sulfonic acid (TAPS), and sodium acetate with 0.1 M NaCl as supporting electrolyte (HEPES, MES, CHES, TAPS, and sodium acetate from Sigma and USB, NaCl from VWR) adjusted to the desired pH with NaOH or HCl. Gas mixtures were purchased from Praxair. Enzyme films were prepared by first abrading the graphite surface with fine emery paper (Norton, P500J) followed by briefly polishing the electrode with a 1 μm aqueous alumina (Buehler) slurry and thorough sonication in water with rinsing. A 25 μL enzyme solution (~0.3 mg/mL) was then pipetted onto the electrode surface and left undisturbed for 120 s, followed by removal of the enzyme solution by pipet and

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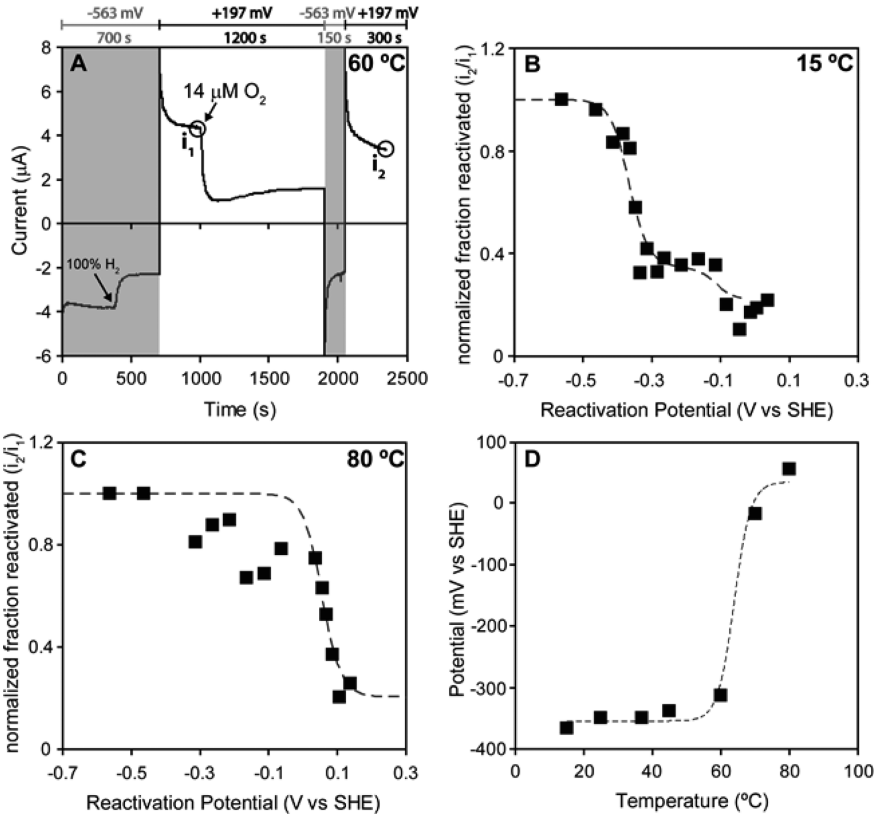

Figure 1. *Pf*SHI reacts with O_2 to form two inactive states that are distinguished by the electrochemical potential required to reactivate them. (A) Current−time trace for a chronoamperometry experiment evaluating impact of oxygen and reduction potential on the catalytic activity of *Pf*SHI. Potentials and durations of the electrochemical steps are noted above the figure. Gray or white background denotes whether the potential of the experiment was more reducing or more oxidizing than the hydrogen couple, respectively. Changes in the gas composition in the experimental apparatus are indicated by arrows. The times at which the currents in other panels were evaluated are indicated by circles. Introduction of H_2 is continuous, but O_2 was introduced via transient injection. Control experiments indicate that after injection, 200 s are required for the O_2 concentration to decrease to an undetectable level. The experimental temperature is 60 °C, and the electrode rotation rate is 3600 rpm. To probe the thermodynamics of reductive reactivation of aerobically inactivated enzyme, the potential of the 150 s reductive pulse was varied. (B) and (C) show the fraction of the H_2 oxidation activity preceding initial exposure to O_2 that was recovered following the reactivation (i_2/i_1) as a function of this reducing potential at 15 °C (B) and 80 °C (C). Data are normalized such that the most highly active sample after reactivation has a fraction reactivated value of 1. The dotted lines (B and C) are fits of the data to a linear combination of two Nernstian processes. (D) shows the dependence of the lower of these two reduction potentials on temperature. The dotted line shows the best fit to a sigmoidal model.

rinsing of the electrode surface with water. All electrochemical experiments were conducted using a three-electrode system consisting of a pyrolytic graphite rotating disk working electrode, a platinum wire auxiliary electrode and a Ag/AgCl reference electrode. The electrode rotation rate was 3600 rpm and for cyclic voltammetry experiments, the potential scan rate was 10 mV s^{-1} unless otherwise indicated. Temperature-controlled experiments were conducted using a water-jacketed electrochemical cell connected to a temperature-controlled water bath. The electrochemical data were analyzed with SOAS, an electrochemical data analysis program downloadable on the web.[73] The $K_m^{H_2}$ values for *Pf*SHI over a range of temperatures were determined using the method described by Léger et al.[74]

RESULTS

Hydrogen Oxidation by *Pf*SHI Displays Oxygen Tolerance. *Pf*SHI can be adsorbed to a pyrolytic graphite electrode and both hydrogen oxidation and proton reduction activity are observed. Chronoamperometry experiments (Figure 1A) were used to probe the effects of oxygen on the hydrogen-oxidation activity of *Pf*SHI and the dependence of reactivation from an inactive, oxidized state on electrochemical potential. Figure 1A shows that exposure to oxygen of a *Pf*SHI film oxidizing H_2 at +197 mV results in an immediate decrease in the catalytic current. Importantly, although the catalytic current drops dramatically, it does not reach zero. Instead it decreases to a stable lower value and then slowly increases as the oxygen diffuses out of the electrochemical cell, indicating spontaneous regeneration of catalytic activity. This behavior is in stark contrast to that of standard [NiFe]-hydrogenases that exhibit a sharp drop to zero without any subsequent increase in activity (see, for example, Figure 5 in ref 70).

Reactivation Following Brief Exposure to Oxygen Reveals Two Inactive States. Standard [NiFe]-hydrogenases are oxidatively inactivated to two distinct states that are distinguished by their kinetics of reactivation: Ni-A and Ni-B. Although Ni-B is reactivated on the time scale of seconds to minutes following a return to reductive conditions, Ni-A requires reduction for tens of minutes before regaining activity.[11,24−30] On the other hand, oxygen-tolerant enzymes have been shown to form only one inactive state, akin to Ni-B. We have shown previously that the oxygen-sensitive *Syn*SH inactivates to form two states, both of which can be *quickly* reactivated.[70] To probe the inactive states formed by *Pf*SHI, we explored its ability to be reductively reactivated following exposure to oxygen.

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As shown in Figure 1A, following brief exposure of the enzyme to 14 μM oxygen, the potential of the electrochemical experiment was dropped to more reducing values for 150 s to initiate reactivation. The potential was then returned to +197 mV to observe the resulting hydrogen oxidizing activity. Figure 1B shows that at 15 °C the extent of reactivation (i_2/i_1, where i_2 is the current 300 s after reactivation and i_1 is the current preceding exposure to O_2) of the enzyme depends on the potential of the reductive pulse in a Nernstian fashion. At less reducing reactivation potentials, less catalytic activity was regenerated. At more reductive potentials, more activity was regained. This suggests that reactivation is a reduction process.

The reactivation data shown in Figure 1B could be fit to a linear combination of two one-electron Nernstian processes. Because of the oxygen tolerance of *Pf*SHI, even at the highest reactivation potentials, some H_2 oxidation activity remained. Thus, to facilitate fitting the data, each data set was normalized into the range 0−1; i.e., the lowest value is zero and highest value is 1, according to the following formula:

$$\mathrm{NV} = \frac{(V - V_{\min})}{(V_{\max} - V_{\min})} \tag{1}$$

where NV is the normalized value, V is the unnormalized fraction reactivated value, $V_{\min}$ is the minimum measured fraction reactivated value (at high potentials) and $V_{\max}$ is the maximum measured fraction reactivated value (at low potentials). The data were then fit to a linear combination of two, one-electron Nernstian processes according to the following equation:

$$\mathrm{NV} = Pf\mathrm{SHI}_{\mathrm{low}}\left(\frac{1}{1 + e^{\left(\frac{nF}{RT}(E - E_{\mathrm{low}})\right)}}\right) + Pf\mathrm{SHI}_{\mathrm{high}}\left(\frac{1}{1 + e^{\left(\frac{nF}{RT}(E - E_{\mathrm{high}})\right)}}\right) \tag{2}$$

where $Pf\mathrm{SHI}_{\mathrm{low}}$ is the fraction (expressed as a decimal) of the adsorbed enzyme present in the low potential reactivating state, $Pf\mathrm{SHI}_{\mathrm{high}}$ is the fraction (expressed as a decimal) of the adsorbed enzyme present in the high potential reactivating state, E_{low} is the apparent reduction potential of the low potential reactivating state, E_{high} is the apparent reduction potential of the high potential reactivating state, n is the number of electrons in the redox process and was fixed at 1, F is the Faraday constant, R is the ideal gas constant, and T is the temperature in Kelvin. We note that this model ostensibly has four variable parameters, but since we have normalized the data set in the range 0−1, the proportions of enzyme in each of the two states must sum to 1. Thus, these two variables are not independent, and the data was fit using only three variables. The best fit at 15 °C yields apparent reduction potentials of −366 mV and 116 mV with 83% and 17% of the inactive enzyme in each state, respectively.

Temperature Dependence of Reactivation. Since the electrocatalytic activity of *Pf*SHI is very sensitive to temperature, experiments were conducted over a range of temperatures between 15 and 80 °C to investigate the effect of temperature on the inactivation/reactivation of *Pf*SHI. First, it is worth noting that, within error, the $K_m^{H_2}$ of *Pf*SHI, 44 μM, is unchanged from 25 to 60 °C (Table S1). Furthermore, the concentration of hydrogen in solution at all temperatures in this study is in the range of 400−800 μM, i.e., an order of magnitude higher than the $K_m^{H_2}$ value. Thus, the H_2 concentration should be sufficient to saturate the active site in all experiments. Figure 1C shows representative data derived from a collection of chronoamperometry experiments obtained at 80 °C. Figures S2 and S3 present analogous data at 25 and 60 °C, respectively. At all temperatures, the extent of reactivation depends on the potential of the reductive pulse in a Nernstian fashion. However, fits reveal a shift in the apparent reduction potentials of reactivation toward less reducing values at higher temperature. In fact, at temperatures ≥37 °C, E_{high} is shifted outside the range of potentials probed in this experiment and so its value cannot be estimated. Table 1 lists the reduction potentials and proportions of each state formed for all temperatures investigated. For temperatures at which both states can be characterized, the proportions of the two states formed are relatively constant. Unfortunately, this only includes data at 15 and 25 °C, and we have been careful not to overinterpret.

Table 1. Reduction Potentials and Proportions of Each Inactive State Formed for All Temperatures Investigated

temperature (° C)	potential of low-potential inactive state (mV)	proportion of enzyme in low-potential inactive state (%)	potential of high-potential inactive state (mV)	proportion of enzyme in high-potential inactive state
15	−366	83	−116	16.6
25	−350	82	−105	17.6
37	−350	N/A	N/A	N/A
45	−339	N/A	N/A	N/A
60	−313	N/A	N/A	N/A
70	−18	N/A	N/A	N/A
80	+55	N/A	N/A	N/A

Although E_{high} could not be determined over the entire temperature range of these experiments, Figure 1D shows the dependence of E_{low} on temperature. It is immediately clear that there is a particularly large shift to more positive potentials between 60 and 80 °C. Essentially, above 60 °C, significantly less negative potentials are required to reactivate aerobically inactivated *Pf*SHI. Quantitatively, the apparent reactivation potentials as a function of temperature can be fit by a sigmoidal equation with the following form:

$$E_{\mathrm{low}}(T) = E_{\mathrm{low},1} + \frac{(E_{\mathrm{low,h}} - E_{\mathrm{low},1})}{(1 + e^{(T - T_0)/w})} \tag{3}$$

$E_{\mathrm{low}}(T)$ corresponds to the value of E_{low} at the temperature T in Kelvin. Four variable parameters were used to fit the data: (1) $E_{\mathrm{low,h}}$, the value in mV vs SHE that E_{low} approaches at high temperature; (2) $E_{\mathrm{low,l}}$, the value in mV vs SHE that E_{low} approaches at low temperatures; (3) T_0, the temperature in Kelvin at which $E_{\mathrm{low}}(T)$ is the average of $E_{\mathrm{low,h}}$ and $E_{\mathrm{low,l}}$ and (4) w, a dimensionless width parameter describing how quickly the values shift between the two limits. The data are best fit with $E_{\mathrm{low,h}}$ of 35 mV, $E_{\mathrm{low,l}}$ of −355 mV, T_0 of 64 °C, and w of 2.4. It is important to note that as an apparent reduction potential E_{low} has no inherent thermodynamic meaning. Instead, the shift in E_{low} with temperature may be an indication of an increase in the rate of reactivation at higher temperatures.

H_2 Oxidation during Prolonged Oxygen Exposure. In addition to probing the response of *Pf*SHI to *transient* exposure to oxygen, we have also investigated the ability of the enzyme to perform catalysis in response to *prolonged* exposure to oxygen. Figure 2 shows chronoamperometric traces demon-

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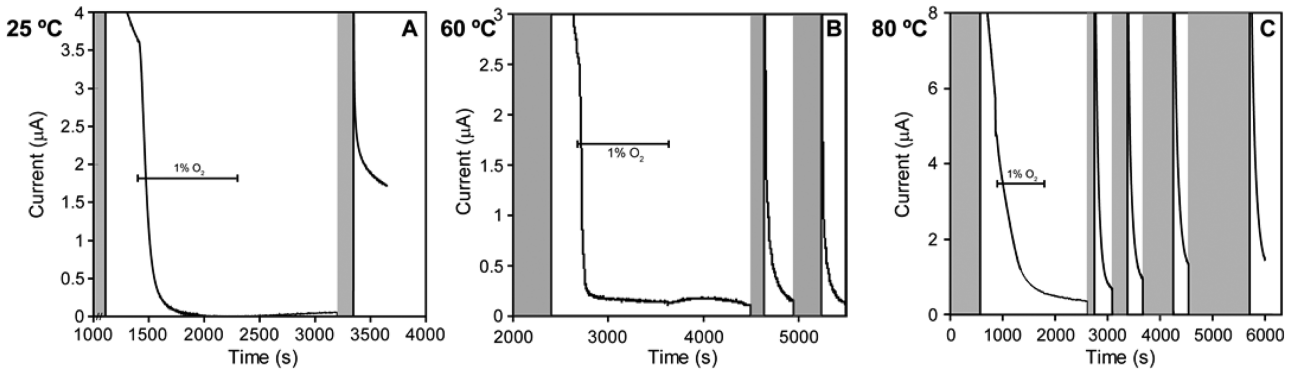

Figure 2. Hydrogen oxidation activity under prolonged O_2 exposure at 25 °C (A), 60 °C (B) and 80 °C (C). Gray panels indicate periods where the electrode was held at reducing (−563 mV) potential and white panels indicate periods where the electrode was held at oxidizing (+197 mV) potentials. Unless otherwise indicated, the electrochemical cell is under 100% H_2. At the time indicated by the bracketed line, the gas in the electrochemical cell was switched to 1% O_2 in 99% H_2 while simultaneously injecting an air-saturated buffer into the electrochemical cell for a final O_2 concentration of 14 μM. Aerobic inactivation of the enzyme was observed in the form of decreasing H_2-oxidation current.

strating the impact of constant exposure to 1% oxygen for 15 min on electrocatalytic hydrogen oxidation by *Pf*SHI at 25 °C, 60 and 80 °C. Initially, the hydrogen oxidation activity was monitored at +197 mV under an atmosphere of 100% H_2. After establishing the level of catalytic activity, the gas was switched to 1% O_2 in 99% N_2, and air-saturated buffer was simultaneously injected into the cell to achieve a final oxygen concentration of 14 μM. Exposure to 1% O_2 was continued for 900 s at which time the atmosphere was returned to 100% H_2. In addition to being exposed to oxygen for a longer period in this experiment, the enzyme is also exposed to oxidizing conditions for a longer interval than during the transient exposure experiments. Figure 2 shows that the response to oxygen is temperature-dependent. At low temperature (25 °C), the hydrogen oxidation catalytic activity drops quickly (~250 s) to zero during the oxygen exposure, and the immobilized enzyme remains inactive until returned to anaerobic conditions (Figure 2A). Following removal of oxygen from the cell to regain anaerobic but oxidizing conditions, a small amount of catalytic activity (~2%) is spontaneously regained without exposing the enzyme to more reducing conditions. On the other hand, at 60 °C, an intermediate temperature, prolonged exposure to oxygen results in a rapid decrease in catalytic activity to a nonzero value (Figure 2B). Over many independent experiments, up to 8% of the enzyme's initial activity was retained after 900 s of exposure to 1% oxygen at 60 °C. When oxygen is removed from the cell to regain anaerobicity, there is also a slight reactivation of the enzyme. However, over longer time periods, (greater than 4000 s in Figure 2B), there is another decrease in activity. This is likely the result of anaerobic, oxidative inactivation. Finally, at 80 °C, the inactivation of *Pf*SHI by oxygen occurs on a much longer time scale. Even after 900 s of exposure at 80 °C there is still detectable hydrogen oxidation activity. Essentially, at 80 °C, a lower, steady state catalytic activity is attained during the prolonged exposure to oxygen; the enzyme is not completely inactivated. When oxygen is removed from the experiment, the activity simply continues at the new value without considerable further inactivation or reactivation. For a collection of three independent experiments at 80 °C, the final activity after exposure to oxygen was in the range of 7−32% of the initial activity under anaerobic conditions (Figure 2C). Thus, we conclude that the proportion of *Pf*SHI that remains active after prolonged exposure to oxygen is directly related to the temperature of the experiment. More experiments will be necessary, however, to quantify this correlation. It is worth noting that the final concentration of oxygen in the solution decreases with temperature (Table S1 and Figure S1): 12.7 μM at 25 °C, 8.8 μM at 60 °C and 8.1 μM at 80 °C. However, since the greatest difference in activity is observed between 60 and 80 °C, we believe the changes are a result of temperature not the change in oxygen concentration.

To characterize the properties of the inactive states formed by prolonged exposure to oxygen, the films inactivated by oxygen exposure were reduced at −563 mV, and the recovered hydrogen oxidation activity at +197 mV was subsequently monitored. Like the inactivation, the properties of reactivation are highly dependent on temperature. At 25 °C, as already described for the transiently aerobic experiments, only a brief, i.e., 2.5 min, excursion to reducing potentials (shown as a gray bar in Figure 2A) was sufficient to observe maximal reactivation. Approximately 49% of the catalytic activity observed prior to exposure to oxygen could be regenerated by this short reductive excursion, and longer exposure did not increase the activity of the film. However, as shown in Figure 2C, at 80 °C only minimal reactivation was observed following brief reductive reactivation. See, for example, data between 2800 and 3100 s. To establish whether enzymatic activity could be recovered by longer exposures to reducing conditions, a series of longer reductive pulses were also used to probe reactivation. Maximal reactivation, circa 30−35% of the preoxygen exposure activity level, was observed only after 37.5 min of low-potential reactivation. This behavior is in contrast to both experiments in the sustained presence of oxygen at low temperature and experiments with transient exposure to oxygen at high temperatures. Thus, the slowly reactivating inactive state formed at high temperature appears to require prolonged exposure to oxygen or highly oxidizing conditions to induce formation.

Finally, anaerobic oxidative inactivation of the enzyme is also clearly observable in these results as a decrease in the oxidation activity even under anaerobic conditions. For example, the exponential decreases in the activities shown in the second and third white panels of Figure 2B and 2C are characteristic of anaerobic, oxidative inactivation. Figure S4 also shows that this anaerobic oxidative inactivation can be observed for films that have never been exposed to oxygen, and that these films does not show any appreciable permanent film loss (due to enzyme

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denaturation or detachment from the electrode) over a time scale of thousands of seconds at 80 °C.

DISCUSSION

Current understanding of oxygen tolerance in [NiFe]-hydrogenases has been informed largely by electrochemical data in concert with EPR, FTIR and X-ray crystallographic studies.[6] While both oxygen-tolerant and oxygen-sensitive group 1 [NiFe]-hydrogenases have been extensively studied using electrochemical methods, this is the first electrochemical characterization of the reactions with oxygen of an oxygen-tolerant, group 3 [NiFe]-hydrogenase containing both hydrogenase and diaphorase components. It is important to consider oxygen-tolerant group 3 enzymes separately because there is no indication that they contain a [4Fe3S] proximal cluster, a component hypothesized to be essential for oxygen tolerance in group 1 enzymes.[32] Complete, heteromultimeric SH complexes have proven difficult to isolate, with heterogeneous preparations often plagued by variable activities or instability.[62,75−81] *Pf*SHI is a particularly appealing enzyme to use for these studies because it comes from a hyperthermophilic organism, making it more stable than most SHs. In addition, affinity-tagged forms that allow facile purification have been successfully homogeneously overexpressed in *Pf*, allowing future access to site-directed mutant enzymes.[71,72,82]

Electrocatalytic Hydrogen Oxidation by *Pf*SHI Is Oxygen-Tolerant. The effect of oxygen on the electrocatalytic activity of *Pf*SHI is different from that of all other types of [NiFe]-hydrogenases studied to date. We have shown herein that *Pf*SHI adsorbed at a graphite electrode is oxygen-tolerant; i.e., it maintains a nonzero fraction of its hydrogen-oxidizing activity in the presence of oxygen. Furthermore, even without exposing the sample to reducing conditions, a small fraction of the lost activity is spontaneously regained upon removal of oxygen from the electrochemical experiment. By comparison, both group 1 and group 3 oxygen-sensitive hydrogenases rapidly lose all activity upon exposure to oxygen and do not regain catalytic activity without exposure of the enzyme to reducing conditions.[70,83] The holoenzyme (tetrameric) form of oxygen-tolerant *Re*SH has not been electrochemically characterized, but electrocatalytic activity of its dimeric subcomplex, *Re*HoxHY, was shown to be quickly and completely inactivated by oxygen (Figure 7 in ref 62). The response of *Pf*SHI to exposure to oxygen is not only different from oxygen-sensitive enzymes but also from that of prototypical, oxygen-tolerant group 1 [NiFe]-hydrogenases. For example, both *E. coli* Hyd1 (Figure 5 in ref 55) and Hase I from *Aquifex aeolicus* (Figure 6 in ref 33) maintain a significantly higher fraction of their oxidizing activity upon exposure to oxygen than *Pf*SHI *and* spontaneously regain most of their activity upon removal of the oxygen from the experiment.

The effect of oxygen on the hydrogen oxidation activity of *Pf*SHI is most similar not to another wild-type hydrogenase but to oxygen-tolerant mutants of *Df*Hase.[57,83,84] Wild-type *Df*Hase is completely unable to recover from transient oxygen exposure at oxidizing potentials, but mutations of V74 confer the ability to oxidatively regenerate up to 60% of H_2-oxidation activity at 40 °C.[57] Electrochemical experiments conducted with *Pf*SHI under similar conditions, i.e., 45 °C, pH 6.4, (Figure 3), demonstrate recovery of 25% of its H_2 oxidation activity at oxidizing potential, which closely resembles the O_2 tolerance behavior of the V74P mutant. The results from the *Df*Hase are important as they demonstrate that an oxygen-sensitive enzyme can be made oxygen-tolerant and that a *range* of oxygen tolerances can be established via mutagenesis. The *Pf*SHI results expand these conclusions by showing that a similar spectrum of oxygen tolerance already exists for wild-type [NiFe]-hydrogenases; *Pf*SHI is somewhere in the middle of this natural spectrum. Thus, oxygen tolerance should not be thought of as a dichotomous, but rather as a continuous, property, and we propose the use of the expression "oxygen-resilient" for enzymes that fall in the middle of the spectrum.

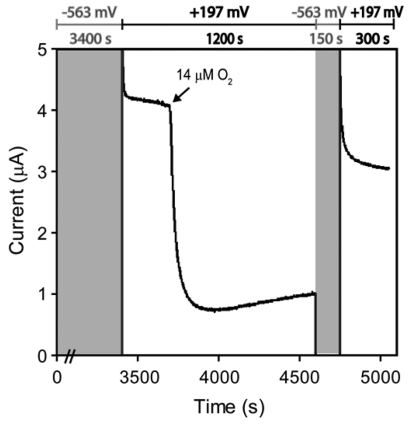

Figure 3. Potential step experiment at 45 °C demonstrating hydrogen oxidizing activity after transient oxygen exposure. Experiment performed at 45 °C under 100% H_2 in a 75 mM mixed buffer at pH 6.4 with an electrode rotation rate of 3600 rpm. Potentials and duration of each step are indicated above the figure. At t = 3700 s, an aliquot of air-saturated buffer was injected into the cell for an initial O_2 concentration of 14 μM, as indicated by the arrow. The oxygen then spontaneously diffuses out of the cell.

Aerobic Inactivation of *Pf*SHI Results in Formation of Three Inactive States. Oxygen tolerance in group 1 [NiFe]-hydrogenases is thought to be achieved by exclusive formation of the Ni-B state upon reaction of the active site with oxygen.[34,36,55,85] See Figure 4A for a summary of the oxidized states of standard [NiFe]-hydrogenases; the oxygen-tolerant enzymes are not believed to form the Ni-A and Ni-SU states. However, our studies of the reactivation of oxygen-inactivated *Pf*SHI show that it is oxygen-tolerant *and* forms at least three, distinct oxygen-inactivated states. These states have different reactivation kinetics, thermodynamics, or, perhaps even more

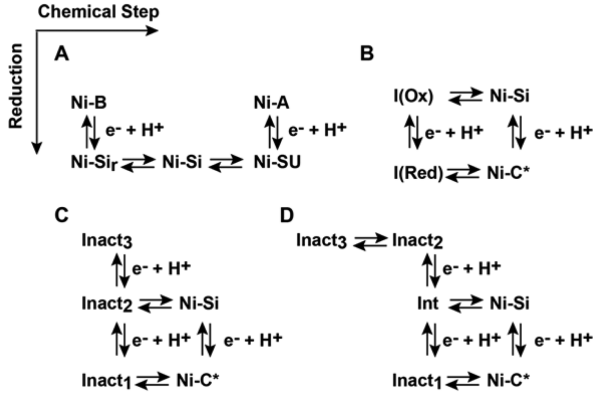

Figure 4. Oxidation schemes for (A) standard [NiFe]-hydrogenases, (B) [NiFeSe]-hydrogenases as proposed in ref 86 and (C) and (D) *Pf*SHI based on data in this work. Additional catalytically active states are not shown.

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likely, both. Transient exposure to oxygen results in formation of only two states, both reactivated relatively quickly (<150 s), but at different apparent reduction potentials. As demonstrated previously, variations in apparent reduction potentials can arise from differences in reactivation kinetics.[86] For example, the species reactivated at higher apparent potential may simply be reactivated more rapidly than the species reactivated at lower apparent potentials. Sustained exposure to oxygen is necessary to produce the third state, which can only be reactivated on a much longer time scales (ca. 30 min). We note that the inactivation electrochemistry experiments expose the enzyme not only to oxygen but also to oxidizing conditions and these, not oxygen itself, may be the trigger required for formation of the inactive states. Further experiments will be necessary to map exhaustively the conditions that can produce each state.

We also evaluated the reactions of *Pf*SHI with oxygen over the range 15−80 °C. At higher temperatures, the apparent potential necessary for reactivation is less negative (Figure 1D); i.e., the active site is easier to reduce. This is an "apparent" reduction potential because we have not demonstrated that it is a thermodynamic parameter via simultaneous fitting of the data using a physical model. Nonetheless, we can conclude that it is easier to reactivate *Pf*SHI at higher temperatures either because the reduction potential of the reactivation transition is less negative or because the kinetics of reactivation are markedly faster (or both). The latter suggests that the rate-limiting step of reactivation is strongly temperature-dependent. One possible explanation is that reactivation is not simply an electron transfer reaction but relies on a rate-limiting, large-scale protein motion. With that in mind, other [NiFe]-hydrogenases might also be more oxygen-tolerant if they could be studied at higher temperatures, and it may be interesting to compare the properties of other [NiFe]-hydrogenases from (hyper)-thermophilic sources with those of *Pf*SHI.

Comparison of Electrochemically Observed Inactive States with Spectroscopic Information. The spectroscopic properties of *Pf*SHI and other [NiFe]-hydrogenases may offer clues to the chemical identities of the *Pf*SHI inactivate states and the possible mechanisms of its oxygen tolerance. Oxygen-sensitive group 1 hydrogenases are inactivated by oxygen or oxidizing conditions to two paramagnetic, Ni(III) S = 1/2 inactive states: Ni-B (also known as Ni-ready) and Ni-A (also known as Ni-unready) (Figure 4A). Ni-B samples can be quickly reactivated by reduction. In contrast, Ni-A is defined not only by a characteristic EPR signal but also by a requirement for exposure of the inactivated enzyme to reducing conditions for extended periods of time (often tens of minutes, see refs 87 and 88) before catalytic activity is regained. Oxygen-tolerant group 1 enzymes are thought to attain oxygen tolerance by exclusive formation of the Ni-B state, and the proximal [4Fe3S] cluster is hypothesized to provide extra reducing equivalents necessary to prevent formation of Ni-A.

FTIR and EPR spectroscopic characterization of group 3 enzymes is less complete than that of the group 1 enzymes, and has not yet led to a consensus description of whether the oxidatively inactivated states differ significantly between these two enzyme types. FTIR and EPR studies have only been reported for three SHs: *Syn*SH, *Re*SH and *Pf*SH1. Using phenazine methosulfate (E_0' = +60 mV) as oxidant, EPR signals for three distinct oxidized forms of *Pf*SHI were reported, referred to as Ni-Ox^{resting}, Ni-Ox^{highT} and Ni-Ox^{lowT}.[89] However, a second study using thionine (E_0' = +11 mV) as oxidant, did not detect any rhombic Ni(III) EPR signals.[90] Similarly, Ni(III) EPR signals associated with the air-oxidized forms of *Syn*SH and *Re*SH were not detected, suggesting that the inactive forms of group 3 enzymes are electronically distinct from those of group 1.[61,91] On the other hand, FTIR investigations of air-oxidized samples of all three enzymes, including *Pf*SHI, identified a single inactive state referred to a Ni$_r$-B-like without a corresponding Ni(III) EPR signal.[61,91,92] It remains unclear whether this species is EPR-silent because it is formally Ni(II) or owing to spin couplings with other paramagnetic centers.

Regardless of the spectroscopic data from the group 3 enzymes, the kinetic properties of the electrochemically produced inactive forms of *Pf*SHI are not consistent with formation of the classical Ni-A and Ni-B states. Furthermore, the Ni$_r$-B-like state detected for all SHs characterized to date is not sufficient alone to explain all of the electrochemically detected states of oxidized *Pf*SHI. Considering only the transient exposure of the enzyme to oxygen, the inactive states formed are very similar to those detected electrochemically for the SH from *Synechocystis* sp. PCC 6803 and the [NiFeSe]-hydrogenase from *Desulfomicrobium baculatum*.[70,93] Léger and co-workers have recently characterized the oxidative inactivation of the [NiFeSe]-hydrogenase from *Desulfovibrio vulgaris* Hildenborough and shown that it too forms two inactive states that are reactivated on relatively short time scales.[86] Perhaps more importantly, that work demonstrates that both inactivate states are formed under anaerobic conditions. They also propose a model (reproduced here in part as Figure 4B) in which one of the inactive states is at the same redox level as the catalytically active Ni−SI state and the other, in a break with conventional models, at the more reduced redox level of Ni−C*, a more reduced state of the catalytic cycle.

Figure 4C shows a model that connects the inactive forms of *Pf*SHI to its catalytic intermediates (Ni−SI and Ni−C*). This model expands that described by Léger and co-workers while taking into account both the electrochemical data described herein as well as previous EPR spectroscopic studies. The central square, including Ni−C*, Ni−Si and the inactive forms 1 and 2, mirrors the model of Léger and co-workers. All of these states are more reduced than the canonical inactive states Ni-A and Ni-B (Figure 4A). We imagine this may explain why both inactive states are quickly reactivated under reducing conditions as we have observed electrochemically. This unusual pair of relatively reduced inactive states is also consistent with previously inexplicable results from Hagen and co-workers.[89] They observed that *Pf*SHI Ni−C was transformed to Ni-Ox^{lowT} under oxidative but low temperature conditions without any detectable intermediates. Furthermore, by heating, Ni-Ox^{lowT} could be converted to Ni-Ox^{HighT}. This topology of conversions suggests that Ni-Ox^{lowT} and Ni-Ox^{highT} are Inact$_1$ and Inact$_2$, respectively. Our model differs from Léger's in the addition of the third inactive state at a more oxidizing level. This state has been added to explain our observations following prolonged exposured of *Pf*SHI to oxygen at high temperatures. We hypothesize that the slowly reactivating state formed under these conditions is the same as the Ni-Ox^{resting} state observed via Ni EPR signals by Hagen and co-workers under highly oxidizing conditions. Thus, we have assigned it as more oxidized than the other inactive states (Inact$_3$). Hagen and co-workers showed that Ni-Ox^{resting} is transformed to Ni-Ox^{highT} by anaerobic incubation at high temperature, and this is consistent with formation of Inact$_3$ via oxidation of Inact$_2$, as shown in our model. Hagen's results also suggest that interconversion of the two states is slow at low temperature,

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consistent with our observation that $Inact_3$ is only observed at high temperatures. Importantly, this could also explain why a state like $Inact_3$ has not been detected for other [NiFe]-hydrogenases studied to date.

There are two more aspects of the spectroscopic data for *Pf*SHI that are not explained by the model in Figure 4C. Hagen and co-workers identified all three of the states we are referring to as $Inact_1$, $Inact_2$ and $Inact_3$ via Ni EPR signals. However, our model places Inact2 at the same level as Ni−SI, meaning it would be expected to be a Ni^{II} species without detectable EPR signals. The basic model could be expanded to take this into account by introducing an EPR-silent intermediate as shown in Figure 4D. Such a model is consistent with our results if we assume that it is only transiently populated such that it is not detectable on the electrochemical time scale. Finally, Hagen and co-workers actually identified each of their oxidized forms as a mixture of "ready" and "unready" signals; i.e., they hypothesized six states formed under oxidizing conditions. We hope that future spectroscopic and electrochemical studies under precisely defined conditions may explain this last observation and provide further tests of the models we have presented here.

Mechanism of Oxygen Tolerance and Structure of Inactive States. Finally, we turn to the question of the mechanism of oxygen tolerance in *Pf*SHI and the structures of the three inactive states formed. The primary sequence of its small subunit (that harbors the three FeS clusters) does not contain the additional cysteine residues required for formation of the [4Fe3S] cluster of oxygen-tolerant group 1 [NiFe]-hydrogenases, making the presence of such a cluster improbable. Similarly, the electrocatalytic and EPR properties of *Pf*SHI are different from the classical, oxygen-tolerant group 1 [NiFe]-hydrogenases, suggesting that electronic differences may play a role in the intermediate oxygen tolerance and formation of noncanonical inactive states. Two alternative structural hypotheses for oxygen tolerance have recently emerged. First, Lenz and co-workers have recently suggested that the oxygen tolerance of *Re*SH is a result of destabilization of Ni-A by promoting sulfoxygenation of a bridging sulfur ligand.[64] Second, the structure of the V74C mutant of *Df*Hase, a variant with marked oxygen tolerance, has also revealed unexpected structural differences in the Ni first coordination sphere. Instead of coordination via four standard cysteine thiolates, the Ni ligands are modeled as the terminal cysteine thiolates in combination with a bridging SH- ligand and a mainchain carboxamido N atom.[58] The amide coordination, also found in Ni superoxide dismutase, is hypothesized to stabilize the Ni(II) state and could also facilitate unusual electronic coupling with the proximal [4Fe-4S] cluster. Electrochemistry does not provide any direct evidence to suggest whether one of these unusual active site modifications can form in *Pf*SHI. However, it is tempting to hypothesize that one or more of the inactive states observed electrochemically features an unexpected active site modification and that these variations also explain the unusual variation of spectroscopic states seen in SHs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b07680.

Additional electrochemical data and concentrations of hydrogen and oxygen in solution at various temperatures. (PDF)

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Notes

The authors declare no competing financial interest.

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