II.G.5 Electrochemical Characterization of the Oxygen-Tolerant Soluble Hydrogenase I from *Pyrococcus furiosus*

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Objectives

The long term goal of this project is to characterize the reactivity of soluble [NiFe]-hydrogenases (SH) as a model for energetically relevant multielectron redox catalysis and to understand what factors control bias towards hydrogen production (or oxidation) and competing reactions with oxygen.

Technical Barriers

Although membrane bound [NiFe]-hydrogenases (MBH) have been extensively characterized via electrochemical methods, soluble [NiFe]-hydrogenases have remained largely unstudied due to difficulty in obtaining stable enzyme samples and functional attachment to electrode surfaces.

Abstract

The soluble hydrogenase I from *Pyrococcus furiosus* (SHI) is the first oxygen-tolerant soluble hydrogenase to be electrochemically characterized. We demonstrate that the electrocatalytic activity is highly sensitive to temperature with the ratio of proton reduction activity to hydrogen oxidation activity shifting dramatically in favor of proton reduction with increased temperature. Similarly, reactions of the active site with oxygen are dependent both on the length of time the enzyme is exposed to oxygen and the temperature of the reaction. We show that SHI is an oxygen-tolerant electrocatalyst, but its catalytic properties are different from the more commonly studied membrane bound [NiFe]-hydrogenases and the mechanisms of oxygen tolerance are likely different.

Progress Report

Pyrococcus furiosus is a hyperthermophilic, strictly anaerobic archaeon that grows optimally at 100 ºC by fermentation of carbohydrates. It produces three [NiFe]-hydrogenases: two soluble group 3 enzymes (SHI and SHII)

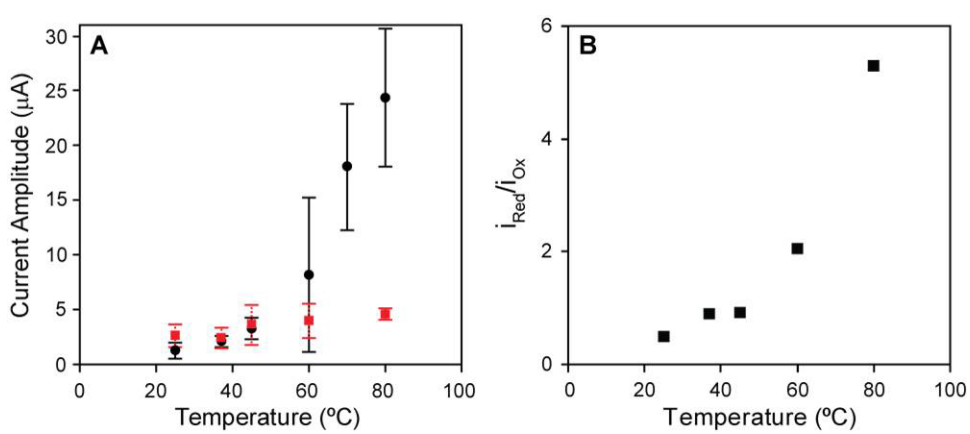

FIGURE 1. Temperature Dependence of (●) H^+ reduction and (●) hydrogen oxidation catalytic currents and the ratio of the two activities from adsorbed *Pf*SHI. Experiments were performed in a mixed buffer with pH = 6.5 at the indicated temperatures with electrode rotation at 2000 rpm.

and a group 4 H_2-evolving membrane-bound hydrogenase (MBH). SHI is a heterotetrameric enzyme believed to produce hydrogen physiologically.

Protein film electrochemistry (PFE) is a technique in which a redox protein is functionally adsorbed to an electrode surface and electron transfer can be observed as current without the addition of a chemical mediator. This method allows exquisite control of electrochemical potential and has proven invaluable in the study of group 1 membrane-bound hydrogenases. However, the reactions of an oxygen-tolerant group 3 SH have not been characterized eletrochemically. Herein, we describe the characterization of SHI using PFE.

Electrocatalysis by SHI immobilized at graphite: catalytic bias

SHI is functional as both a hydrogen oxidation and a proton reduction catalyst when immobilized at a pyrolytic graphite electrode. Solution assays of SHI have shown that the activity is highly dependent on temperature. Thus the electrocatalytic activity of adsorbed SHI was probed at pH 6.5 at temperature in the range 25-80 ºC. Figure 1 shows the electrocatalytic currents, proportional to turnover frequency, for both proton reduction and hydrogen oxidation as well as the ratio of the two catalytic activities. Both hydrogen oxidation and proton reduction activities

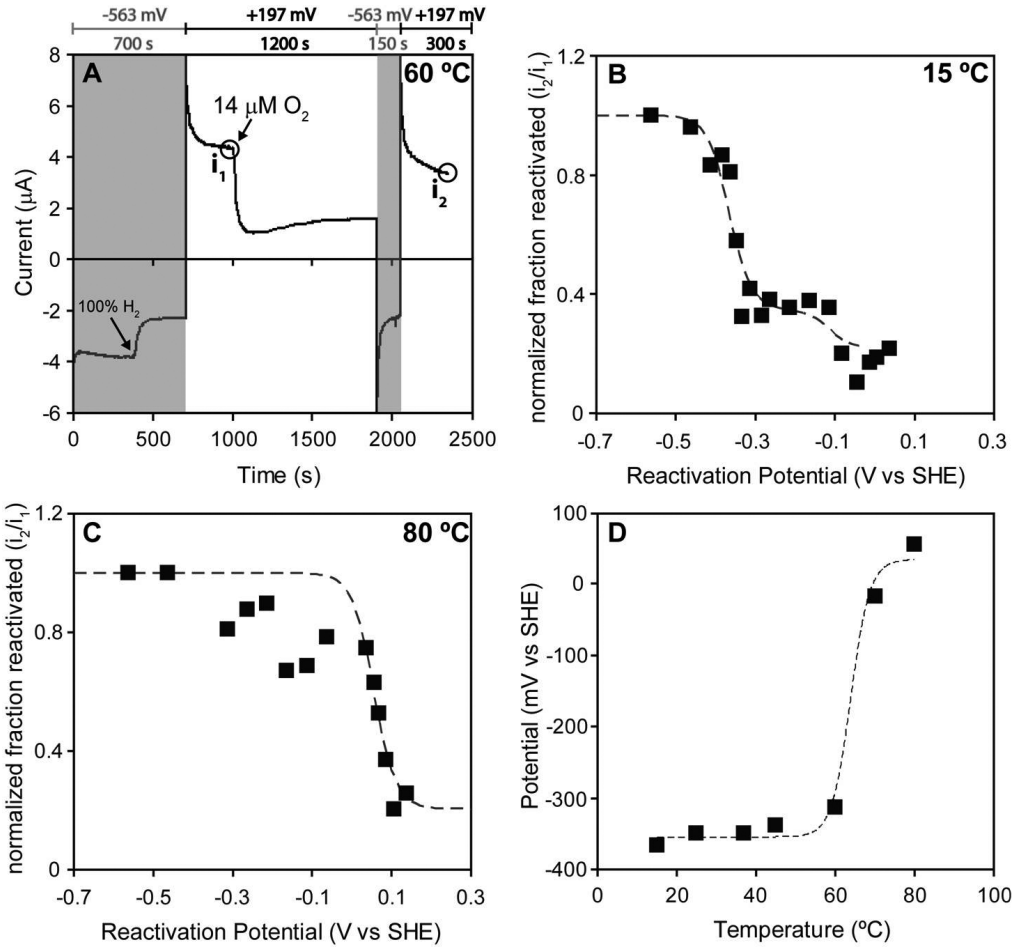

FIGURE 2. SHI reacts with O_2 to form two inactive states that are distinguished by the electrochemical potential required to reactivate them. These reactivation profiles shift to less reducing potentials with increasing temperature. (A) Current-time trace for a chronoamperometry experiment evaluating the catalytic activity of *Pf*SHI in the presence of different gases and at different reduction potentials. The potentials and durations of the various electrochemical steps are noted above the figure. Grey background denotes portions of the experiment in which the potential was more reducing than the hydrogen couple and white background indicates periods in which the working electrode potential was more oxidizing than the hydrogen couple. Changes in the gas composition in the experimental apparatus are indicated by arrows on the current trace. The times at which the currents in later panels were evaluated are indicated by circles. Introduction of hydrogen is continuous, but oxygen was introduced via injection so that it is only transiently present in solution. Control experiments indicate that after injection, 200 s are required for the oxygen concentration to decrease to an undetectable level.

increase directly with temperature. However, the increase of proton reduction activity is far more dramatic. The result of this uneven response is a shift in the catalytic bias of the enzyme towards a marked preference for proton reduction at higher temperatures. Typically, [NiFe]-hydrogenases have been thought of as "uptake" hydrogenases, meaning their activity is higher for the oxidation direction. These results demonstrate that this is clearly an oversimplified understanding of their activities.

Reactions of SHI with oxygen

Prototypical [NiFe]-hydrogenases are known to be reversibly inactivated by both oxidative anaerobic and aerobic conditions. In constrast, so-called oxygen-tolerant [NiFe]-hydrogenases are defined by their ability to maintain some level of hydrogen oxidation activity in the presence of oxygen. Chronamperometry experiments were used to probe the impact of oxygen on the hydrogen oxidation activity of SHI. As shown in Figure 2A, exposure of an enzyme film to oxygen results in an immediate decrease in hydrogen oxidation activity. Although the current decrease is dramatic, the activity drops to a non-zero level. This is in contrast to standard [NiFe]-hydrogenases that are completely inactived by transient exposure to oxygen. Furthermore, after the experimental conditions were returned to anaerobiosis, the immobilized sample spontaneously regained some catalytic activity. Similarly, when the potential was dropped to more reducing conditions, the activity of the enzyme was regained on a timescale of seconds. This reductive reactivation is far faster than that of aerobically inactivated MBHs. Panel 2D shows that the potential of this reduction reactivation, like catalytic bias, was also very sensitive to temperature. It appears that it is thermodynamically easier to reactivate inactive enzyme at more physiologically relevant temperatures.

In addtion to probing the response of SHI to transient exposure to oxygen, we also evaluated the impact of prolonged exposure to oxygen on hydrogen oxidation activity. Figure 3A shows a chronoamperometric trace demonstrating the impact of constant exposure over 900 s to 1% oxygen on hydrogen oxidation catalysis. The catalytic activity drops to zero until the experiment is returned to anaerobic conditions. Following removal of oxygen, a small amount (<2%) of catalytic activity is regained without exposure to reducing conditions. As in the experiments with transient exposure to oxygen, larger fractions of the activity could be recovered by short exposures to reducing conditions. This activity, like the other activities of SHI, also showed a marked temperature dependence (Figure 3B). At higher temperatures, longer exposure to oxygen was necessary to observe complete inactivation. Suprisingly, the inactive form also required longer reduction to regain catalytic activity. This suggests that two inactive forms are generated upon long-term exposure to oxygen, and the ratio of the populations of the two states is highly sensitive to temperature.

Future Directions

SHI clearly demonstrates oxygen-tolerance and is the first oxygen-tolerance soluble hydrogenase to be electrochemically characterize. Oxygen-tolerance in MBHs is thought to arise from the presence of an unusual [4Fe3S] cluster, but phylogenetic analysis suggests that SHs do not possess such a cluster. Thus the explanation of the reactivity of SHI must lie elsewhere. Future studies will target the molecule details of this mechanism.

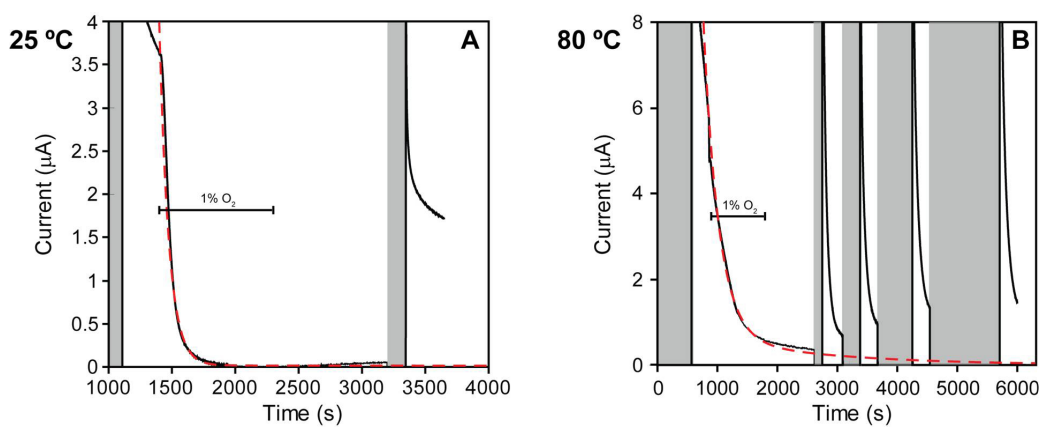

FIGURE 3. Hydrogen oxidation activity under prolonged O_2 exposure at 25 ºC (A) and 80 ºC (B). Gray panels in **(A)** and **(B)** indicate periods where the electrode was held at reducing (-563 mV) potential and white panels indicate periods where the electrode was held at oxidizing (+197 mV) potentials. Unless otherwise indicated, the electrochemical cell is under 100% H_2. At 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.