Hydrogenases

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Hydrogen-Activating Enzymes: Activity Does Not Correlate with Oxygen Sensitivity**

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Hydrogenases (H_2ases) catalyze a reaction that is essential in the energetics of many bacteria and has promising technological applications: the reversible conversion between dihydrogen and protons. These enzymes are studied in various contexts, including bioenergetics and inorganic catalysis, but the main motivation is that the knowledge we shall acquire by studying them will prove useful for designing the catalysts we need to produce hydrogen from water in a clean process and to oxidize it in affordable fuel cells. However, the fact that H_2ases are inhibited by O_2 hinders technological developments and much effort has been devoted to understanding the molecular basis of O_2 sensitivity.[1,2] Herein, we show that a very active H_2ase, which was believed to be highly oxygen sensitive, actually reacts very slowly with O_2 as a result of simultaneous reactions which we were able to resolve and quantify.

Hydrogen-activating enzymes are classified as NiFe, Fe, and FeFe H_2ases according to the metal content of their active site. Clostridial-type FeFe H_2ases house four iron–sulfur (FeS) clusters in addition to the active-site "H-cluster". *Clostridium acetobutylicum* (*Ca*) FeFe H_2ase oxidizes H_2 faster[3] than the thoroughly characterized enzyme from *Desulfovibrio desulfuricans* (*Dd*), which has a similar structure except that the electron-transfer (ET) chain consists of two FeS clusters rather than four (Figure 1).

We have used the procedure described in reference [3] to express homologously and purify by affinity chromatography to apparent homogeneity (as determined by sodium dodecylsulfate PAGE and elemental analysis) the strep-tagged FeFe H_2ase (HydA1) from *Ca* (see the Supporting Information).

Figure 1. Structures of *D. desulfuricans (Dd)* H_2ase (Protein databank (PDB) file: 1hfe; dark gray) and *C. pasteurianum* H_2ase (CpI; PDB file: 1feh; light gray). The latter is highly homologous to *C. acetobutylicum (Ca)* H_2ase (HydA1).[4]

This enzyme spontaneously adsorbs onto graphite in a configuration that allows direct ET to and from the electrode. The steady-state voltammograms in Figure 2 show that *Ca* H_2ase efficiently catalyzes both H_2 oxidation and production. The reductive current is independent of H_2 pressure, which shows that H^+ reduction is not inhibited by the product, H_2. The Michaelis constant (K_m) for H_2 is high (about 0.8 atm or 0.6 mM). The activity decreases at potentials above 80 mV versus the SHE at pH 7, a result that reveals the reversible anaerobic formation of the inactive state of the H-cluster

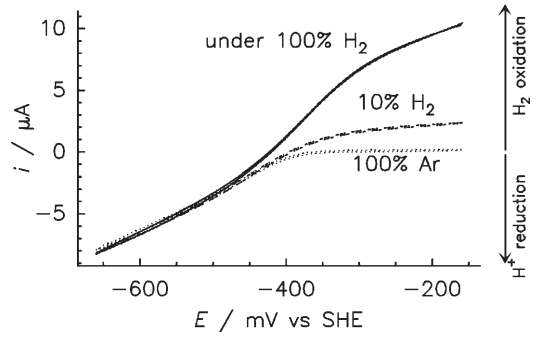

Figure 2. Steady-state cyclic voltammograms for *Ca* H_2ase adsorbed onto graphite. Conditions: pH 7, $T = 30\,^{\circ}C$, $V = 20\ mV s^{-1}$, $\omega = 2$ krpm.

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called H_{ox}^{inact}. All of the above observations are similar to those made with *Dd* H_2ase.[2] However, we will show that great differences appear when the enzymes are exposed to oxygen.

We have developed a method to characterize the reaction of redox enzymes with gaseous inhibitors;[5,6] this method consists of poising the enzyme-coated electrode at a given potential and then injecting into the electrochemical cell an aliquot of solution saturated with inhibitor while the cell is continuously degassed by bubbling through a different gas, for example, H_2. The transient current (that is, the activity) is monitored while the concentration of inhibitor is decreasing. With this decay being exactly exponential, the quantitative analysis of the data is relatively straightforward.

In Figure 3, each column depicts a different experiment. The lower panels show the changes in activity that follow the addition of inhibitors as depicted in the upper panels. The cell

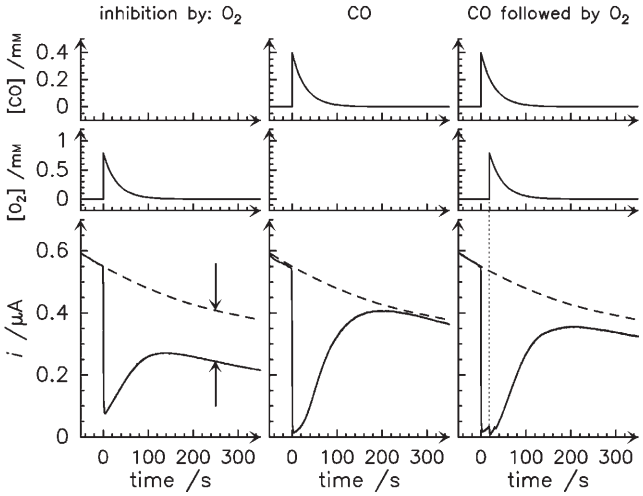

Figure 3. Activity transients following the addition of CO and/or O_2 under H_2 (1 atm). Conditions: pH 7, $T = 30\,°C$, $E = 200$ mV, $\omega = 2$ krpm. The dashed line is a guide for the eye. The current transients in the middle and right columns have been multiplied by 0.83 and 0.95, respectively.

was flushed with H_2 and the electrode was poised at 200 mV; the enzyme slowly inactivates in the absence of inhibitor (at $t < 0$) upon formation of H_{ox}^{inact}. In the experiment shown on the left in Figure 3, the oxygen concentration was stepped to $[O_2]_0 = 0.8$ mM at $t = 0$, which resulted in instant inhibition. Two observations from this experiment contrast with the behavior observed with the enzyme from *Dd*. Firstly, despite the high level of O_2, inhibition is not complete: an O_2 concentration that is ten times lower causes complete inhibition of *Dd* FeFe H_2ase (bottom panel of Figure 5 in reference [7]). Secondly, although a fraction of the activity is irreversibly lost after the oxygen has vanished (at the point marked by arrows), a surprisingly large part of the activity is recovered. When the inhibition was induced by CO (middle column in Figure 3), the irreversible inactivation was not observed, a result indicating that this effect is specific to the reaction with O_2. Hence, oxidative inhibition of *Ca* H_2ase consists of three distinct processes: 1) the anaerobic formation of H_{ox}^{inact} (which is reverted only by reduction),[2] 2) the reversible binding of inhibitor O_2, and 3) an irreversible inactivation by O_2. To determine whether the latter process results from O_2 damaging the electron-transferring FeS clusters or the active site, we ran an experiment where O_2 was added while CO was present (right-hand column in Figure 3). CO is a competitive inhibitor of the enzyme, so it should bind to and protect the active site. The fact that the recovery of activity after aerobic inactivation is greater in this experiment shows that the oxygen damage is due to degradation of the H-cluster rather than the other FeS clusters.

This conclusion is supported by an independent experiment in which the isotope-exchange activity (the formation of HD and H_2 from D_2)[8] was monitored in the presence of O_2. The H/D activity decreased only 50 and 70% in the presence of 3 or 13 μM O_2, respectively, and it was mostly (at low $[O_2]$) or partly (at high $[O_2]$) recovered when O_2 was removed by adding reduced methyl viologen (Figure 4). Since isotope exchange does not involve ET through the FeS clusters, this

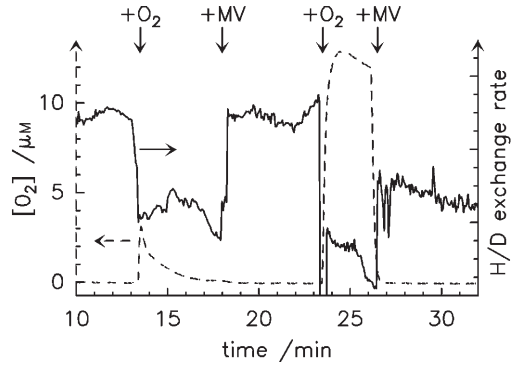

Figure 4. Inhibition of isotope exchange by O_2 in solution at pH 7 and 30 °C. The activity of the sample at time zero was 50 μmol min^{-1} mg^{-1} for both H_2 production and H^+/D_2 exchange. MV: methyl viologen.

shows that O_2 affects the H-cluster. Moreover, since this experiment is carried out in solution, it demonstrates that the oxygen tolerance seen in electrochemistry does not result from the enzyme being adsorbed on (and protected by) the electrode.

Hence, our experiments demonstrate that O_2 inhibition proceeds by reversible formation of an O_2 adduct followed by irreversible transformation of the active site, as depicted in Scheme 1.

$$H_{ox}^{inact} \xleftarrow{k_1} H^{act} \underset{k_{-2}}{\overset{k_2[O_2]}{\rightleftharpoons}} O_2\text{-adduct (inact)} \xrightarrow{k_3} H^{dead}$$

Scheme 1. Oxidative inhibition of *Ca* H_2ase.

We now turn to simulations of chronoamperometric data which allow *quantitative* characterization of the inactivation processes. In Figure 5, current transients resulting from the addition of various amounts of O_2 were fitted to the current equation calculated from Scheme 1, with the exponential decays of O_2 concentration taken into account: $[O_2] =$

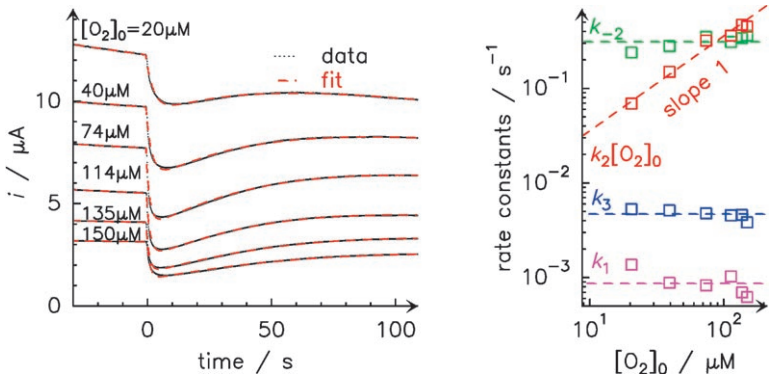

Figure 5. Fit of chronoamperometric data to the solution of the equation described by Scheme 1. The adjusted rate constants are plotted against added O_2 in the right panel. Conditions: 1 bar H_2, pH 7, 30 °C, 2 krpm.

$[O_2]_0\exp(-t/\tau)$. In addition to the value of τ, four rate constants were determined, k_1, $k_2[O_2]_0$, k_{-2}, and k_3, which span three orders of magnitude. Consistently with Scheme 1, the value of $k_2[O_2]_0$ was found to be proportional to the concentration of added O_2, whereas all three other rate constants were independent of $[O_2]_0$. We determined that $k_1 = 10^{-3}\,s^{-1}$, $k_2 = 3.2\,s^{-1}\,mM^{-1}$, $k_{-2} = 0.3\,s^{-1}$, $k_3 = 4\times10^{-3}\,s^{-1}$.

The fact that purification of active clostridial H_2ases requires strictly anaerobic conditions has always been interpreted as revealing the extreme oxygen sensitivity of these enzymes. On the contrary, our data and quantitative analysis show that this enzyme is less sensitive to oxidative inactivation than most other H_2ases:

- We estimate that the rate of anaerobic oxidation ($k_1 = 10^{-3}\,s^{-1}$) is about an order of magnitude slower than that for the enzyme from *Dd*; this inertia is actually detrimental to *Ca* H_2ase since the H_{ox}^{inact} state is protected against O_2 and can be reactivated by reduction.[2]
- Although oxygen irreversibly inactivates the enzyme from *Dd*, it binds *reversibly* to *Ca* H_2ase ($k_{-2}/k_2 = 0.1$ mM). This is usually typical of O_2-resistant enzymes.[2]
- The oxidation that is responsible for irreversibly inactivating the enzyme when it is purified under air is surprisingly slow ($k_3 = 4\times10^{-3}\,s^{-1}$).
- The apparent bimolecular rate constant for the reaction with O_2, $k_2k_3/(k_3+k_{-2}) = 4\times10^{-2}\,s^{-1}\,mM^{-1}$, is much smaller than that for prototypical NiFe H_2ases (32 $s^{-1}\,mM^{-1}$ in reference [5]), which are usually said to be more resistant to O_2.

The molecular basis of this relative resistance must now be determined. The enzymes from *Dd* and *Ca* are very similar in the region surrounding the active site (Figure 1).[4] Yet we have shown that their active sites react very distinctively with O_2. *Ca* H_2ase is one of the very few H_2ases for which efficient molecular-biology tools are now available.[9] The precise characterization of the oxidative reactions described herein will guide our attempts to engineer the enzyme in order to slow further the reactions that make it sensitive to O_2.

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