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Hydrogen-activating Enzymes: Activity Does Not Correlate With Oxygen-Sensitivity

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Protein production and purification.

Clostridium acetobutylicum ATCC 824 contains an FeFe hydrogenase (HydA1) and a putative NiFe hydrogenase, which was revealed by genome sequencing. The FeFe hydrogenase gene is located on the chromosome whereas the NiFe hydrogenase is on a separate megaplasmid (pSOL1). The HydA1 hydrogenase was homologously expressed from the pPHhydA$_{Ca}$-C-tag plasmid in the form of a C-terminal *Strep*-tag tagged protein and purified as described in ref. 3. The purified fraction showed only one band identified as the HydA1-*Strep*-tag protein by immunoblot detection with Strep Tactin HPR conjugate (data not shown). In addition, the iron content of the HydA1-Strep-tag preparation was determined by ICP-AES (ref. 3): the stoichiometric level of iron per hydrogenase molecule was in agreement with the HydA1 content. This elemental analysis also showed that the Ni/Fe ratio was lower than 1/1000 (the Ni content was below the detection limit), demonstrating that our sample was not contaminated by the NiFe hydrogenase. It is not possible to calculate an accurate purification factor of HydA1-*Strep*-tag based on the measurement of the hydrogenase activity at each step of the purification procedure because the initial *C. acetobutylicum* cell-free extract contains two hydrogenases in addition to HydA1-*Strep*-tag (the native HydA1 and the putative NiFe-hydrogenase). From 1 liter of culture, we could routinely obtain 0.1 mg of HydA1 Strep-tagged protein. Aliquots of enzyme solution were frozen at -80°C under a 100% hydrogen atmosphere just after the purification. Once unfrozen, the samples were stored at 4°C in the anaerobic glove box until they were used. The enzyme, freshly purified as described in ref. 3, exhibits a specific H_2 uptake activity of 5.7-7.2 x 10^5 µmol H_2/min/mg at 37°C, pH 7.2. In comparison, the reported Vmax values for H_2 uptake of purified enzymes from *Clostridium pasteurianum* and *Desulfovibrio vulgaris* Hildenborough are 14 000-24 000 and 50 000 µmol H_2/min/mg, respectively (MW Adams and LE Mortenson 1984, J. Biol. Chem., 259:7045-7055; MW Adams 1990, BBA 1020:115-145). In the case of *C. acetobutylicum* HydA1, the number of active enzyme molecules in a sample decreases as a result of aging and/or freezing and thawing (e.g. about half of the overall activity is lost after the enzyme has been kept at -80°C for one month). We have not systematically monitored the change in H_2-oxidation activity over time, but in control experiments carried out over a period of more than 18 months, we have checked that the following parameters were not affected by aging: the Km for H_2 (measured as described in ref. 5), the rates of CO binding and release (measured electrochemically as described in ref. 6), the kinetics of reaction with O_2 (both in electrochemistry and in isotope/exchange assays). Hence the enzyme's properties which we report in the main text are independent of the overall activity and history of the sample.

Isotope exchange

Isotope exchange measurements and rate calculations were carried out essentially as described in ref. [8]. D_2 was bubbled in 1.5mL of 20 mM K-phosphate buffer, pH 7, kept at 30°C in a closed, thermostated vessel. The bottom of the vessel was connected to a mass spectrometer by a polypropylene membrane. Anaeorobiosis was imposed prior to hydrogenase inlet by adding 100 µM reduced methylviologen ($MV^{+\bullet}$). Hydrogenase activity resulted in D/H^+ scrambling and was detected by continuously monitoring the formation of HD and H_2. The H/D exchange rate, taken as a proxy of hydrogenase activity, was defined as the rate $(2d[H_2]/dt + d[HD]/dt)$ normalized by the D content of the hydrogen gas mixture $([D_2]+1/2[HD])/([H_2]+[HD]+[D_2])$ (see ref. [8] for derivation). Oxygen was added in the vessel by injecting 50 or 200 µl aliquots of air-equilibrated water. Anaerobiosis was restored by adding $MV^{+\bullet}$ again.

Direct electrochemistry.

The electrochemical setup and equipment was as described previously (refs. [5,6]). The enzyme was adsorbed onto the pyrolytic graphite edge electrode (geometric area 0.2 cm^2) simply by painting onto the freshly polished surface a dilute solution of enzyme (0.5µM). All experiments were carried out in an anaerobic glove box with residual oxygen content < 0.5ppm. All potentials are quoted against the standard hydrogen reference electrode.

Oxygen was added in the cell by using gas-tight syringes to inject aliquots of buffer solutions saturated with pure oxygen or CO at 30°C. The concentrations were calculated using values of 1.25mM O_2 and 1mM CO under one atmosphere of either gas. The experiments in fig. 3 were carried out by adding 3mL of solution saturated with pure O_2 to 1.75mL of anaerobic buffer (left column), 0.75mL of solution saturated with CO to 1mL of anaerobic buffer (middle column), 0.75mL of CO saturated solution and subsequently 3mL of O_2 saturated solution to 1mL of anaerobic buffer (right column). The experiments in fig 6 were carried out by adding 50 to 500µL of solution saturated with O_2 to about 3mL of buffer under 1 bar H_2.

In these experiments, the actual oxygen (or CO) concentration is not independently measured as a function of time: the initial concentration (just after the addition) is determined as explained above and from the control experiments described in ref. 5, we know that this concentration decreases exactly exponentially with time. The time constant of this decay (which does depend on experimental parameters that are typically not kept constant) is determined *a posteriori* from fitting the chronoamperometric data.

The chronoamperometric data in fig. 5 were modelled by considering that the instant current is proportional to the concentration of H^{act}, which is given by the solution of the following set of differential equations (ODE):

$$\begin{aligned} t < t_0 \qquad & d/dt(\Gamma_{\mathrm{H^{act}}}) = -k_1 \Gamma_{\mathrm{H^{act}}} \\ t > t_0 \quad & d/dt \begin{pmatrix} \Gamma_{\mathrm{H^{act}}} \\ \Gamma_{\mathrm{O_2-adduct}} \end{pmatrix} = \begin{pmatrix} -k_1 - k_2[\mathrm{O_2}]_0 \exp(-\frac{t-t_0}{\tau}) & k_{-2} \\ k_2[\mathrm{O_2}]_0 \exp(-\frac{t-t_0}{\tau}) & -k_{-2} - k_3 \end{pmatrix} \begin{pmatrix} \Gamma_{\mathrm{H^{act}}} \\ \Gamma_{\mathrm{O_2-adduct}} \end{pmatrix} \end{aligned} \qquad (1)$$

where t_0 is the time of injection of O_2, Γ_X is the surface concentration of species X, and the initial condition is $\Gamma_{Hact}=1$. The data were analysed and fit using an in-house program called SOAS, which embeds FFT and fitting routines available on the NETLIB repository (www.netlib.org) and fourth-order Runge-Kutta integrators taken from the Numerical Recipes® handbook (www.nr.com).