Valine-to-Cysteine Mutation Further Increases the Oxygen Tolerance of Escherichia coli NiFe Hydrogenase Hyd-1

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A Valine-to-Cysteine Mutation Further Increases the Oxygen Tolerance of *Escherichia coli* NiFe Hydrogenase Hyd-1

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Abstract: Some NiFe hydrogenases are particularly resistant to O_2, as a result of either the natural presence of a particular FeS cluster or the artificial replacement of a conserved valine residue near the Ni site. We show that the two protective effects can be combined in a single enzyme, by constructing and characterizing the V78C variant of the naturally O_2-tolerant *E. coli* NiFe hydrogenase Hyd-1. We elucidate the effect of the mutation by comparing the kinetics of inhibition by CO and O_2 of a number of wild-type forms and valine-to-cysteine variants of NiFe hydrogenase.

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Hydrogenases are the enzymes that oxidize and produce H_2. They are all inhibited by O_2, although the extent, kinetics and nature of the inactivation vary greatly. Here we discuss the hydrogenases that bear a NiFe dinuclear active site. The so-called "O_2-tolerant" NiFe hydrogenases embed an unusual [4Fe-3S] cluster near the active site, as part of a redox chain that mediates intramolecular electron-transfer. The general consensus is that this cluster causes resistance to O_2 [1,2] (see, however, ref. 3). On the other hand, the so-called "standard", O_2-sensitive NiFe hydrogenase from *Desulfovibrio fructosovorans* (Hyn) can be made significantly resistant to O_2 by replacing a conserved valine residue near the active site [4] (figure 1) with a cystein. [5] (Having any hydrophilic residue at that position in *Df* Hyn enhances the rate of reactivation after oxidative inactivation [6] and this position also determines the intramolecular diffusion rates. [4]) *Escherichia coli* expresses one hydrogenase from each group, Hyd-1 and Hyd-2, respectively. [1,7,8]

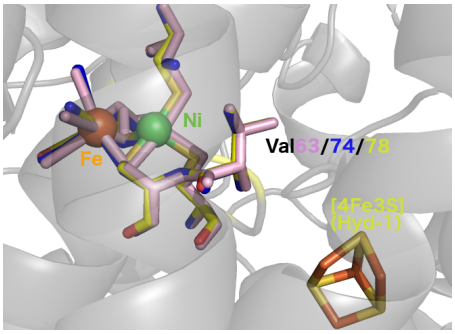

Figure 1. Overlay of the structures of the active site of *E. coli* Hyd-1 NiFe hydrogenase (yellow), *E. coli* Hyd-2 (pink) and *D. fructosovorans* Hyn (blue), together with the conserved V74/V78 residue, and the proximal [4Fe3S] cluster of Hyd-1. pdb accession codes 3UQY, [9] 6EHQ [10] and 1YQW. [11]

Lukey and coworkers have reported the production of recombinant Hyd-1 and Hyd-2 enzymes of *E. coli*, with the small subunit bearing a C-terminal His_6-tag, from engineered operons on the chromosome. [7] On our side, we produced Hyd-1 (wild-type and V78C) and Hyd-2 enzymes from plasmids in the *E. coli* FTD147 (DE3) strain carrying chromosomal in-frame deletions of the genes encoding the large subunits of hydrogenase-1, -2, and -3. [12–14] Recombinant Hyd-1 hydrogenases were produced as a dimeric and soluble form, consisting of the large (HyaB) and the small subunits (HyaA) only (figure S1A). The C-terminal membrane-anchoring hydrophobic helix of HyaA was replaced with a Streptag II. Recombinant Hyd-2 hydrogenase consists of HybO (the small subunit), whose C-terminal transmembrane domain was cleaved by a treatment with trypsin, and HybC (the large subunit) bearing a N-terminal His_6-tag (figure S1B). The enzymes Hyd1 and Hyd2 characterized here appear to have greater and lower activity, respectively, that those prepared using the methods in 7. See in SI more information about the production and activity of these enzymes, and a comparison with previously published activity values.

We compared the properties of wild-type recombinant (WT) Hyd-1 and the V78C variant using direct electrochemistry, [15,16] whereby the enzyme is adsorbed onto a rotating electrode and any change in current reports on a change in turnover frequency, resulting e.g. from exposure to inhibitors (CO or O_2) and/or redox-driven (in)activation.

Figure 2 compares the response of WT Hyd-1 and V78C to transient exposures to O_2. We produced "bursts" of O_2 by injecting in the electrochemical cell small amounts of O_2-saturated solution while simultaneously flushing the solution with H_2. In both cases the inhibition is reversed by removing O_2: the enzymes reactivate after exposure to O_2, even under the very oxidizing conditions used here to prevent the direct reduction of O_2 on the electrode (+140 mV). All things being equal, the V78C variant is less inhibited than the WT enzyme.

We determined the rate constants of inactivation ($k_i^{O_2}$) and reactivation ($k_a^{O_2}$) in this experiment by fitting [18] a model [4] which assumes that the O_2-adduct can also irreversibly inactivate (with 1st-order rate constant k_3):

$$\text{active} \underset{k_a}{\overset{k_i^{O_2} \times [O_2]}{\rightleftharpoons}} \text{adduct} \xrightarrow{k_3} \text{dead end} \qquad (1)$$

The rate constants in Table 1 show that V78C inactivates about 5-times more slowly than the WT; the mutation has a smaller effect on the reactivation rate. Whereas the inactivation of the WT enzyme is fully reversible ($k_3 = 0$), we found that the V78C data are best described using a small value of $k_3 \approx 0.001\ s^{-1}$.

To determine whether the V78C-induced decrease in the rate of reaction with O_2 is due to the mutation hindering the diffusion along the gas channel, we compared the rates of inhibition of the enzymes by CO, which reports on the rate of intramolecular diffusion. [4,16,19] Figure 3 shows the response of

Table 1. Kinetic properties of NiFe hydrogenases

enzyme	$k_i^{O_2}$ ($mM^{-1}s^{-1}$)	$k_a^{O_2}$ (s^{-1}) at 140 mV	$k_{in}^{app,CO}$ ($mM^{-1}s^{-1}$)	k_{in}^{CO} ($mM^{-1}s^{-1}$)	k_{out}^{CO} (s^{-1})	K_I^{CO} (μM)	K_m (mbar H_2)[i]	refs
Ec Hyd-1 WT	20±3[a]	0.16±0.02[a]	1.5±0.7[e]	376±176[e]	0.9±0.2[e]	2.4[e]	4±2[e]	this work
Ec Hyd-1 V78C	4±1[a]	0.24±0.09[a]	1.5±0.6[e]	16±6[e]	0.6±0.2[e]	38[e]	100±7[e]	this work
Ec Hyd-2 WT	5±1[a]	0.002±0.001[a]	>20[e]	>1000[e]	>2[e]	0.9[e]	19±6[e]	this work
Df Hyn WT	30[b]	n/a		63000[e], 2000[f]	500[e], 2[f]	0.65[e]	10±5[h]	4,5,19,20
Df Hyn V74C	10[c]	0.001[c]		120±50[g]	1.5±0.5[g]		610±320[h]	5,6
Aa WT	2.5[d]	0.004[d]	n/a	n/a	n/a	n/a	n/d	21

Experimental conditions: [a]40°C, pH 6, +140 mV; [b]40°C, pH 7, +200 mV, no significant differences were found between pH 7 and 6 or between +200 mV and +140 mV;[20] [c]40°C, pH 5.5, +140 mV; [d]40°C, pH 7, +140 mV; [e]40°C, pH 6, -60 mV; [f]9°C, pH 7, -160 mV; [g]9°C, pH 5.5, -160 mV; [h]pH 7, -160 mV, no significant effect of varying the temperature was detected. [i] 1 mbar of H_2 is equivalent to 0.8 μM of dissolved H_2.[22]

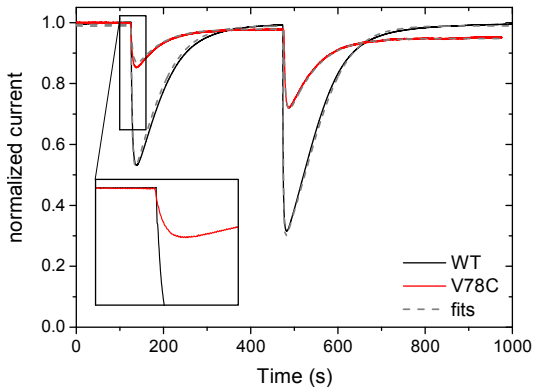

Figure 2. Aerobic inhibition of Hyd-1 WT (black) and V78C (red) *Ec* Hyd-1. The current was divided by the baseline to remove the effect of film loss and anaerobic inactivation.[17] The grey dashed line is the best fit of the model in eq. 1.[4] [O_2] = 8 and 20 μM injected at 125 and 475 s respectively, E = +140 mV *vs* SHE, ω = 3000 rpm, 1 bar H_2, 40°C, pH 6.

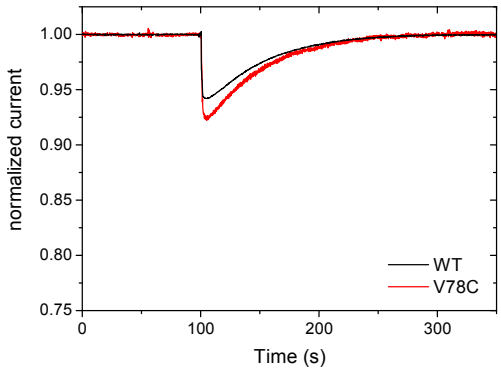

Figure 3. Effect of transient exposure to CO on the catalytic current of Hyd-1 WT (black) and V78C (red). [CO] = 38 μM, E = −60 mV *vs* SHE, ω = 3000 rpm, 1 bar H_2, 40°C, pH 6.

the two enzymes to a transient exposure to CO. We measured the rates of CO binding ($k_{in}^{app,CO}$) and release (k_{out}^{CO}) listed Table 1 by fitting the following model

$$\text{active} \underset{k_{out}^{CO}}{\overset{k_{in}^{app,CO} \times [CO]}{\rightleftharpoons}} \text{inactive} \qquad (2)$$

Since CO competes with H_2,[4,20] the apparent rate of CO binding $k_{in}^{app,CO}$ is lower than the rate of binding in the absence of H_2 by a factor $(1 + [H_2]/K_m)$. We measured the Michaelis constants (K_m) by examining how the steady-state H_2-oxidation current depends on H_2 concentration: the results in figure 4 show that the mutation greatly increases K_m. We confirmed the low value of K_m for Hyd-1 WT by running experiments such as those described in ref. 4, where the current is measured as a function of time while the H_2 concentration is decreasing (see SI figure S2). The V78C mutation increases the value of K_m 25-fold (Table 1).[23]

We used the measured values of K_m to correct the apparent rates of CO inhibition and obtain the true values of k_{in}^{CO}.[4,20] The results in table 1 show that the rate of CO binding for the V78C variant is one order of magnitude slower than for the WT, which would have escaped detection if we had not measured the K_m values.

The effect of the mutation on the kinetics of reversible oxidative inactivation can be assessed under anaerobic conditions in voltammetric experiments, where the electrode potential is swept and the slow, redox-driven conversion between active and inactive states produces a hysteresis.[24] Reactivation occurs on the sweep to low potential because the rate of reactivation increases as the electrode potential decreases. The "switch potential" E_{sw} where the activity returns on the downward trace, is the potential below which the reactivation becomes faster than the voltammetric time scale ($F\nu/RT$, where ν is the scan rate): the faster the reactivation or the slower the scan rate, the greater E_{sw}.[6,25] The V78C mutation shifts E_{sw} up by about 25 mV, and thus increases the rate of reactivation after anaerobic oxidative inactivation by a factor of about 2. We calculate from figure 5 the rates of reactivation at E_{sw} = 140 mV after anaerobic inactivation to be $F\nu/RT$ =0.10 s^{-1} for WT Hyd-1, hence 0.19 s^{-1} for V78C. These rates are similar to those measured for the reactivation after aerobic inactivation (0.16 and 0.24 s^{-1} in Table 1): in both forms of the enzyme, the species formed under oxidative aerobic and anaerobic conditions reactivate at the same rate, and thus are likely to be the same.

Table 1 also shows kinetic parameters measured with Hyd-2 (SI figures S3–S5). Its inhibition by CO is fast at 40°C and only lower values of the binding and release rates could be estimated, but their ratio K_I^{CO} is easily measured and well defined.

Overall, the above data provide a fresh perspective on the reactivity of NiFe hydrogenases with inhibitors, and new evidence that it is strongly affected by the protein matrix.

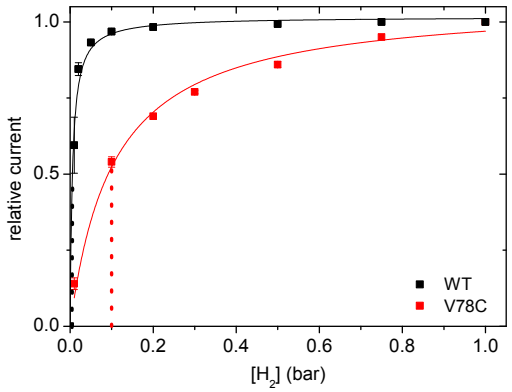

Figure 4. Steady-state current against $[H_2]$ from chronoamperometry experiments with Hyd-1 WT (black) and V78C (red). The dotted lines mark the Michaelis constants. $E = -60$ mV *vs* SHE, $\omega = 3000$ rpm, $T = 40°C$, pH 6.

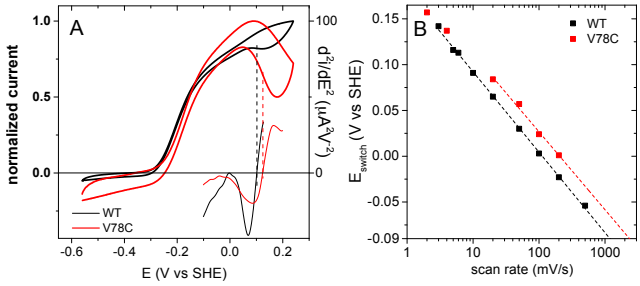

Figure 5. Effect of the V78C mutation on the anaerobic inactivation and reactivation of Hyd-1. Panel A: Cyclic voltammograms (thick lines, left axis) at 5 mV/s of Hyd-1 WT (black) and V78C (red) adsorbed at a rotating disk electrode and their second derivative (thin lines, right axis). The E_{sw} values are indicated by dashed lines. Panel B: Influence of scan rate on E_{sw} for Hyd-1 WT (black) and V78C (red). The slopes of the dashed lines are 85 and 89 mV/decade (V78C and WT, respectively).[26] 1 bar H_2, $T = 40°C$, mixed buffer pH 6.

Escherichia coli Hyd-1 is much less inhibited by CO than Hyd-2.[7] This may be a general property of O_2-resistant NiFe hydrogenases: the enzyme from *Aquifex aeolicus* is apparently not inhibited by CO (unpublished results of ours) and according to a FTIR investigation, interacts weakly with CO.[27] Here we reported inhibition constants (K_I's), but also, and for the first time, the rates of CO binding and release for an O_2-resistant NiFe hydrogenase (Table 1). The most significant difference between Hyd-1 and Hyd-2 is actually the *kinetics* of inhibition, which is much slower for the O_2-resistant *Ec* Hyd-1 than for standard hydrogenases (*Ec* Hyd-2 or *Df* Hyn).

Previously, based on the characterization of a large series of site-directed variants of *Df* Hyn, we have shown that the rate of inhibition by CO is a proxy of the rate of intramolecular diffusion (in this enzyme, CO-binding at the active site is very fast and does not limit the rate of inhibition). To support the hypothesis that this is also true for *Ec* Hyd-1, we note that the effect of the V-to-C mutation is about the same in *Df* Hyn and *Ec* Hyd-1: a 15 to 25-fold decrease in k_{in}^{CO}, a 25 to 60-fold increase in K_m, suggesting that the V-to-C mutations slow the rates of intramolecular diffusion of CO, H_2 (and probably also O_2) in both enzymes. If so, the data in Table 1 show that something specific about the structure of O_2 resistant NiFe hydrogenases significantly slows the diffusion of small ligands. Simulations have been performed to probe this process in both standard[28,29] and O_2-resistant[30–32] NiFe hydrogenases; it has been proposed that the latter have narrower tunnels with fewer openings, which may indeed contribute to slowing down the intramolecular diffusion of small molecules.

Table 1 shows that the rates of binding and release of CO are much more variable than the rates of O_2-binding, which differ less than ten-fold. Hyd-1 reacts with O_2 much more slowly than with CO, and since there is no reason to assume that these two ligands diffuse in the enzyme at different rates, the difference clearly indicates that intramolecular diffusion is not the rate limiting step of inhibition by O_2. Therefore, the decrease in the rate of reaction with O_2 that results from the V74C in *Df* Hyn or the V78C mutation in *Ec* Hyd-1 does not result from the channel being obstructed and the diffusion hindered, but rather from a change in the reactivity of the active site.

We have observed before that replacing V74 in *Df* Hyn changes the rate of reactivation after oxidative inactivation by orders of magnitude,[6] the reactivation is particularly fast in the V74C variant.[5] A surprise in our study of *Ec* V78C Hyd-1 is that the effect is qualitatively the same as, but quantitatively much smaller than in *Df* Hyn. The data in figure 5 show that the reactivation rate increases only two-fold in *Ec* V78C Hyd-1, compared to 25-fold in *Df* V74C Hyn (data at $E = -90$ mV from ref. 5). But similar to our previous conclusion with *Df* Hyn, we see that in the WT just like in the V78C mutant, the rates of reactivation after aerobic and anaerobic inactivation are the same, suggesting that the species formed under aerobic and anaerobic conditions are the same.[33]

Among NiFe hydrogenases for which electrochemical data are available, the V78C Hyd-1 mutant appears to be the most tolerant towards CO and O_2. The k_{in}^{CO} values of Hyd-1 WT and V78C are, respectively, two and three orders of magnitude smaller than that of *Df* WT (63000 $mM^{-1}s^{-1}$, extrapolated to 40°C). The rate of reaction with O_2 (4 $s^{-1}mM^{-1}$) is in the lower range of the values obtained with NiFe hydrogenases (from 2 to 30 $s^{-1}mM^{-1}$) and the reactivation at 140 mV after exposure to O_2 is about two orders of magnitude faster than the rates found for *Df* V74C, Hyd-2 or the O_2-resistant hydrogenase from *Aa* (see table 1). The reactivation rate constants extrapolated to $E = -90$ mV from the data in figure 5 are 50 and 90 s^{-1} for Hyd-1 WT and V78C, respectively, which is 3 orders of magnitude faster than *Df* WT (0.02 s^{-1} in ref. 5) and also faster than *Df* V74C (0.5 s^{-1})[5] and *Aa* WT (5 s^{-1})[25]).

Our results show that a mutation that makes standard NiFe hydrogenase resist O_2 can be transposed into an enzyme that is already resistant. The effects of the mutation on $k_{in}^{O_2}$, k_{in}^{CO}, and K_m are qualitatively the same in *Df* Hyn and *Ec* Hyd-1, so that the beneficial effect of the mutation improves further the properties of the naturally resistant enzyme. One problem though is the $\sim$ 20 fold lower activity of the V78C mutant compared to Hyd-1 WT. In *Df* Hyn V74C, the residue C74 binds the Ni in the as-prepared enzyme,[34] but a normal coordination can be restored upon reduction.[5] It may be that this perturbed structure is also present in *Ec* Hyd-1 V78C, but too stable to be transformed back to the normal state; indeed, we could not observe with the V78C *Ec* hyd1 variant the reductive activation observed with V74C *Df* Hyn[5].

Associated content

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Authors contributions

The manuscript was written through contributions of all authors.

Supporting Information Available: A SI file is available free of charge: materials and methods (section S1), SDS-PAGE of purified Hyd-1 and Hyd-2 enzymes (Figure S1), measurement of K_m for Hyd-1 WT (Figure S2), measurement of K_m for Hyd-2 (Figure S3), effect of O_2 on Hyd-2 (Figure S4), effect of CO on Hyd-2 (Figure S5). This material is available free of charge via the Internet at `http://pubs.acs.org/`.

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