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Understanding and Tuning the Catalytic Bias of Hydrogenase

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Supporting Information

ABSTRACT: When enzymes are optimized for biotechnological purposes, the goal often is to increase stability or catalytic efficiency. However, many enzymes reversibly convert their substrate and product, and if one is interested in catalysis in only one direction, it may be necessary to prevent the reverse reaction. In other cases, reversibility may be advantageous because only an enzyme that can operate in both directions can turnover at a high rate even under conditions of low thermodynamic driving force. Therefore, understanding the basic mechanisms of reversibility in complex enzymes should help the rational engineering of these proteins. Here, we focus on NiFe hydrogenase, an enzyme that catalyzes H_2 oxidation and production, and we elucidate the mechanism that governs the catalytic bias (the ratio of maximal rates in the two directions). Unexpectedly, we found that this bias is not mainly determined by redox properties of the active site, but rather by steps which occur on sites of the proteins that are remote from the active site. We evidence a novel strategy for tuning the catalytic bias of an oxidoreductase, which consists in modulating the rate of a step that is limiting only in one direction of the reaction, without modifying the properties of the active site.

The four Michaelis parameters (two maximal rates and two values of K_m) which characterize the forward and reverse reactions of a one-substrate one-product enzyme are related to each other and to the equilibrium constant of the reaction by the Haldane equation.[1] The forward and reverse maximal rates sometimes differ so much that certain enzymes were designated as "one-way enzymes".[2] The origin of such kinetic asymmetry, referred to as "catalytic bias", has rarely been investigated. Jencks proposed that directionality may result from the destabilization of the enzyme−substrate complex, which would decrease the energy required to reach the transition state in the forward direction.[2] This "Circe effect" is controversial[3] and has found no echo in the case of oxidoreductases, whose directionality is always discussed by comparing the potential of the substrate/product redox couple with the potential of either the active site or the redox centers of the electron transfer (ET) chain, when there is one (see examples in Supporting Information (SI)).

In trying to explain the catalytic bias from a single property of the enzyme (the potential of either the active site or the ET chain), one implicitly assumes that a single redox step, ET either between the substrate and active site or to/from an electron relay, determines both maximal rates. However, the catalytic cycle of oxidoreductases involves various steps (substrate binding, product release, proton and electron transfers, active-site chemistry) and it may occur that the rate limiting step (rls) is not the same when the enzyme works forward or backward. Two different steps may define the two maximal rates, and their ratio. Demonstrating that this can occur requires that the rls be defined in both directions in a series of variants that exhibit different catalytic preferences. Hereafter, we do so by characterizing a series of *Desulfovibrio fructosovorans* (*Df*) NiFe hydrogenase mutants. Figure 1 shows the structure of the wild type (WT) enzyme and illustrates the idea that its catalytic mechanism involves sites of the protein that are far apart from one another. Previously, we have shown that the WT enzyme catalyzes H_2 production and oxidation at similar maximal rates and that narrowing the substrate channel (Figure 1d) using site directed mutagenesis has no effect on the maximal rate of H_2 oxidation.[4,5] Here we show that these mutations slow H_2 production up to 100-fold. In redox titrations, the active site of the mutants that have little reductive activity behaves as that of the WT enzyme. We use a novel method based on the isotope-exchange assay to determine the rates of H_2 release from the active site to the solvent, and we conclude that this step limits H_2 production whereas H_2 entry does not determine the maximal rate of H_2 oxidation. Conversely, the previous observation[5] that modifying the ET chain selectively slows H_2 oxidation shows that ET limits the rate of H_2 oxidation but not H_2 production.[6] This is the first demonstration, on a specific example, that slowing a step that is rate limiting only when the enzyme works in one direction is a general mechanism for biasing the enzyme in the other

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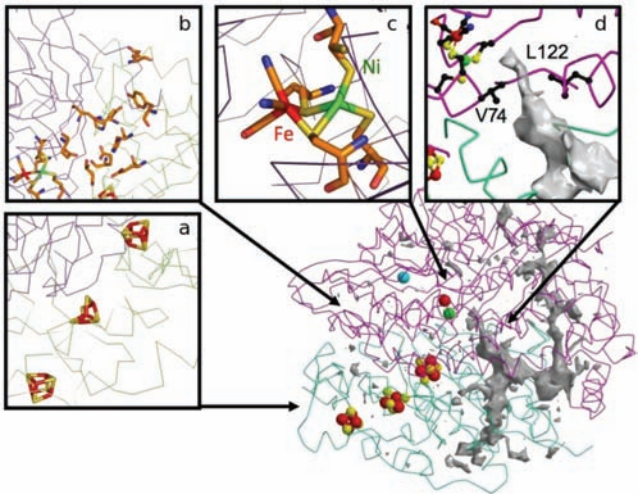

Figure 1. Structure of *Df* NiFe hydrogenase (pdb accession code: 1YQW). Close-ups show the structural elements that are involved in the catalytic cycle: (a) the FeS clusters that wire the active site to the redox partner, (b) a chain of amino acids that are putatively involved in proton transfer,[11] (c) the active site, and (d) the gas channel.

direction, independently of the redox properties of the cofactors.

We prepared the enzyme samples as described previously.[5] H_2 oxidation rates were measured using a spectrophotometric assay at pH 8, 30 °C, with 50 mM oxidized methyl viologen (MV), under 1 atm of H_2.[5] The K_m for H_2 were measured electrochemically.[5] The voltammograms in Figure 2 were

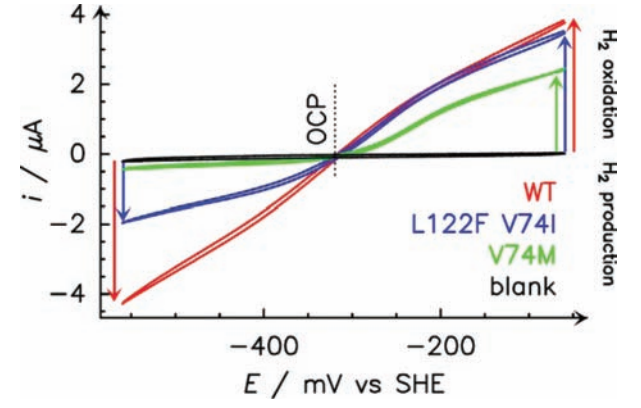

Figure 2. Bidirectional electrocatalysis by the WT (red), L122F-V74I (blue), and V74 M (green) forms of *Df* NiFe hydrogenase. The conditions (10% H_2, pH 5.5, 40 °C, scan rate 10 mV/s, electrode rotation rate 3 krpm) ensure that H_2 oxidation (at high electrode potential) and production (at low potential) are detected in the same experiment. At the open circuit potential (OCP), the rates of oxidation and reduction exactly cancel each other. The blank was recorded with no adsorbed enzyme.

obtained with the enzyme bound to a rotating-disk graphite electrode.[7] H_2 production was followed using mass spectrometry (MS), in a solution initially saturated with Ar, with a saturating concentration of reduced MV (2 mM) at pH 7.2, 30 °C; since H_2 inhibits H_2-production by NiFe hydrogenases,[8] we extrapolated the initial rates using a plot of 1/rate against 1/[H_2]. We performed the H^+/D^+ exchange experiments at pH 7.2, 30 °C,[4] and FTIR titrations at pH 8.[9]

Previous investigations using crystallography and molecular dynamics have predicted that a network of hydrophobic channels guides H_2 to/from the buried active site of *Df* NiFe hydrogenase.[10] Substituting Leu122 and Val74, which shape a bottleneck in the channel (Figure 1d), strongly decreases the rates of intramolecular transport of H_2, CO, and O_2 in both directions.[4,5] The decrease of the H_2-binding bimolecular rate constant has no effect on the *maximal* rate of H_2 oxidation obtained by extrapolation to infinite concentration of H_2.[5] Furthermore, the mutations considered below moderately increase the Michaelis constant for H_2 (5- to 30-fold for L122F-V74I and V74M, respectively), but the Michaelis constant is small in the WT (~10 matm of H_2) and these mutations decrease less than 2-fold the H_2 oxidation rate under 1 atm of H_2.[5] Hence, substrate binding does not limit H_2 oxidation under 1 atm of H_2 in the WT and in these mutants.

Figure 2 compares electrochemical signals obtained with the WT enzyme and two mutants (L122F-V74I and V74M, pdb 3CUS and 3H3X) attached to rotating-disk graphite electrodes.[7,8,12] Hydrogen oxidation and production are detected as positive and negative currents in a single experiment where the electrode potential is swept across a wide range. The absolute magnitude of the current is meaningless because it is proportional to turnover rate times the *unknown* electroactive coverage, but Figure 2 clearly shows that the mutations decrease the ratio of oxidation over reduction currents: unlike the WT, the mutants are biased toward H_2 *oxidation*. This illustrates that the term "bias" does not refer to "the direction of the reaction", which is imposed by thermodynamics, but to the ratio of rates (here, currents) *measured for the same* reaction *proceeding in opposite directions*. Since the conditions chosen for the two measurements are different, the ratio of rates (i.e., the value of the bias) is not an equilibrium constant.[12] In electrochemical experiments, thermodynamics only forces the "open circuit potential" (OCP) to equate the reduction potential of the H^+/H_2 couple given by the Nernst equation; it is indeed independent of the enzyme (Figure 2).[8,12]

The results of solution assays with oxidized or reduced methyl viologen (MV) quantify the bias of the mutants. Figure 3a shows the maximal rates of H_2 oxidation against the maximal

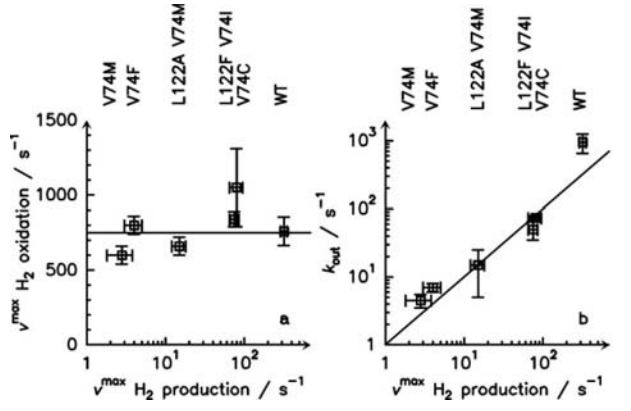

Figure 3. Kinetic properties of the hydrogenase channel mutants. Panel a shows the maximal rate of H_2 oxidation plotted against the maximal rate of H_2 production for six forms of the enzyme and demonstrates that these mutants are biased toward H_2 oxidation. Panel b shows the rates of H_2 release (measured using eq 1 from experiments such as those in Figure 4) against the maximal rates of H_2 production and demonstrates that the former limits the latter.

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rates of H_2 production. The mutants have the same oxidation activity as the WT (only V74C is slightly more active[9]) whereas their maximal H_2 production rates vary by 2 orders of magnitude. The ratio of maximal rates (oxidation over production) ranges from 2.5 for the WT to 200 for the V74M mutant. Note that the exact value of this bias is not an intrinsic property of the enzyme: it depends on which reaction is catalyzed (it is not the same for hydrogenase exchanging electrons with MV, an electrode, or its physiological partner) and on the experimental conditions that are arbitrarily chosen to measure the two maximal rates.

To examine whether the decrease in H_2 production activity was due to a mutation-induced increase in the reduction potential of the active site, we compared the redox properties of the active site in the WT enzyme and the V74C and V74 M mutants, which exhibit intermediate and extreme bias values. Redox titrations of the active sites of these enzymes were monitored by FTIR in a spectroelectrochemical cell as described before.[9] The FTIR signatures of these mutants are similar to those of the WT enzyme. Supplementary Figure S2 shows the redox titration of the CO band of the Ni-C state for the three hydrogenases, from which the reduction potentials of the active site catalytic intermediates could be determined. Table 1 shows that all results are similar. Leucine 122 is more

Table 1. Redox Properties of the WT Enzyme and Two Mutants[a]

enzyme	Ni-A/Ni-SU	Ni-B/Ni-SI	Ni-SI/Ni-C	Ni-C/Ni-R	bias
WT	−195	−175	−330	−430	2.5
V74C	−175	−135	−370	−450	13
V74M	−210	−200	−320	−440	200

[a]Ni-A, Ni-B, and Ni-SU are inactive forms of the enzyme, whereas Ni-SI, Ni-C, and Ni-R are catalytic intermediates.[14] All reduction potentials are in mV vs SHE. Typical errors are ±15 mV. The bias shown here is the ratio of the maximal rates of oxidation over production.

distant from the active site than V74 (Figure 1d); we therefore expect that L122 mutations should have no effect on the active site potential either. This suggests that the low reductive activities of the mutants are not the consequence of the potential of the active site being shifted upward compared to the WT value.

In search of the rate limiting step of H_2 production, we determined the rate constants of H_2 release, under the same conditions as H_2 production, using the isotope exchange assay, where the enzyme transforms D_2 into HD and eventually H_2 using protons from the solvent.[4] The reaction is followed using mass spectrometry (Figure 4). Assuming that all diatomic molecules diffuse to the active site with a bimolecular rate constant k_{in} and from the active site with a first-order rate constant k_{out}, and that H^+/D^+ exchange at the active site proceeds with a first-order rate constant k, we predicted[3] that both the concentration of D_2 and the isotope content $T = [D_2] + [HD]/2$ should decrease exponentially with time, as observed in Figure 4, with rates k_D and k_T, respectively, which depend on k_{in}, k_{out}, k, e_0 (the enzyme concentration), and c_0 (the initial concentration of D_2). We discuss in SI (section S2) the hypothesis that there is no significant isotope effect on k_{in} and k_{out}. The analysis that we previously reported allowed the determination of only the ratio k_{out}/k (ref 4). We now show in SI that under certain conditions, which apply to the case of *Df*

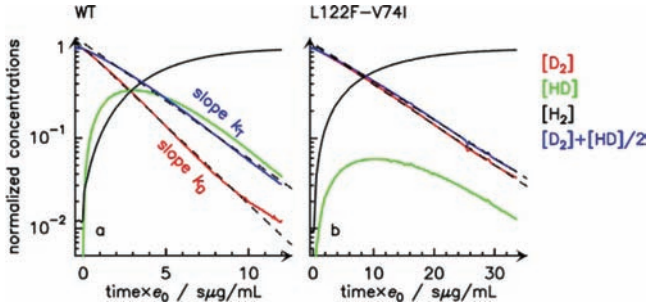

Figure 4. Isotope-exchange assay of the WT (a) and L122F-V74I mutant (b). The changes in concentrations were used to determine k_{out} using eq 1.

NiFe hydrogenase, the rate of H_2 release k_{out} can be deduced from the data using eq 1:

$$k_{out} = \frac{k_D k_T}{2k_T - k_D} \frac{c_0}{e_0} \quad (1)$$

When k_{out}/k is small, little HD is released, $k_D \approx k_T$ (Figure 4b), and $k_{out} \approx k_D c_0/e_0$.

We used this method with all above-mentioned mutants to determine the rates of release of H_2 (k_{out}), which we plotted against the maximal rate of H_2 production in Figure 3b (note the log−log scale). All data points are close to the $y = x$ line, demonstrating that H_2 production is limited by H_2 release from the active site: the mutants have little production activity *because* the channel is obstructed, and H_2-diffusion also determines the rate of H_2-production in the WT enzyme.

Conversely, we previously made the same enzyme a better catalyst of H_2 production than H_2 oxidation by modifying its ET chain.[6] The enzyme has a series of three FeS clusters, which "wires" the active site to the enzyme's redox partner (Figure 1a). The surface-exposed (distal from the active site) [4Fe4S] cluster is coordinated by His184; replacement with Gly184 or Cys184 slows H_2 oxidation 33- and 60-fold, respectively, whereas the rates of H_2 production decrease only 1.3- and 2.1-fold (Table 1 in ref 6). These mutants are biased toward H_2 *production*: both mutations decrease the ratios of maximal rates (oxidation over production) by a factor of ∼27. The H184G mutant has an open coordination site on one Fe ion of the distal cluster; when exogenous imidazole binds to this cluster, the H_2-oxidation rate increases from 3% to 33% of that of the WT, whereas the H_2-production rate is not affected:[6] binding of imidazole to the distal cluster of H184G partially repairs the ET chain and restores the native catalytic bias. It has long been suggested that ET limits the rate of H_2-oxidation in WT NiFe-hydrogenase,[15] and this was recently supported by measurements of ET rates in the enzyme from *D. fructosovorans*.[16] That a modification of the distal cluster biases the enzyme in the direction of H_2 production shows that ET is rate limiting only for H_2-oxidation.

Regarding the WT and mutant hydrogenases discussed here, our data show that H_2-diffusion in the gas channel is the rls of H_2-production but not H_2-oxidation, whereas the latter is limited by ET. Altering one of these steps selectively affects one of the two reactions. We therefore evidence a novel mechanism for tuning the catalytic bias of an oxidoreductase which is independent of the properties of the active site but requires that the rls be different *under the two different sets of conditions that are used to drive catalysis in one direction and the reverse*. This contrasts with all previous explanations of the catalytic bias of

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oxidoreductases (see examples in SI) which proposed that it may be determined by the redox properties of the cofactor(s).

The above results, and previous evidence that enzymes' turnover rates may be limited by steps other than active site chemistry (e.g., proton transfer, electron transfer, substrate release or lid opening[17]), emphasize the need to study all steps of the reaction rather than only active site chemistry. Identifying the rls in a catalytic reaction, keeping in mind that active site chemistry may be fast, is a difficult task. Yet this is a prerequisite if one aims at understanding global kinetic properties, such as rate enhancement, proficiency, or bias.

Hydrogenases could be used as H_2 oxidation or production catalysts in biotechnological devices[18] if the enzymes that can be produced in large amounts were not inhibited by O_2. This has motivated research on the inhibition mechanism, and *Df* NiFe hydrogenase mutants that proved more resistant to O_2 than the WT enzyme have been characterized; this includes the V74C and V74M mutants studied here.[9,19] That mutations which increase O_2 tolerance may also change the catalytic bias of the enzyme will have to be considered in studies which aim at optimizing this biological catalyst. Our results also call for further studies of what defines the catalytic bias toward dihydrogen oxidation[20] or production[21] that has been observed in other native NiFe hydrogenases.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Complete refs 4−6, 9, 16, 19 ; examples of discussions in the literature of what may determine the bias of oxidoreductases ; demonstration of eq 1 ; redox titration curves of the WT and the V74C and V74 M mutants. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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