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New perspectives in hydrogenase direct electrochemistry

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Abstract

Electrochemical studies of hydrogenases, the biological catalysts of H_2 oxidation and production, have proven wrong the old saying that enzymes do not easily transfer electrons to electrodes in the absence of mediators. Many distinct hydrogenases have actually been directly connected to electrodes or particles, for studying their catalytic mechanism or for designing solar fuels catalysts. In this review, we list the electrodes that have proved successful for direct electron transfer to hydrogenases, and we discuss recent results which illustrate new directions in this research field: the study of the biosynthesis of FeFe hydrogenase, the electrochemical characterization of non-standard NiFe- or FeFe hydrogenases, the general discussion of what makes a catalyst better in one particular direction of the reaction, and the elucidation of the molecular mechanism of hydrogenase catalysis by combining electrochemistry and theoretical chemistry, spectroscopy or photochemistry. The electrochemical methods described herein will probably prove useful for studying or using other redox enzymes.

¹ Introduction

 Hydrogenases are the enzymes that oxidize and produce H2. They are classified as NiFe- and FeFe-hydrogenases based on the metal content of their active site. The NiFe active site (fig. 1A) consists of pair of metal ions bridged by the sulfur atoms of two cysteine residues; the Ni ion is also attached to the protein by either two cysteines or one cysteine and one selenocysteine.

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Figure 1: The X-ray structures of two hydrogenases. Panel A shows the active site of NiFe hydrogenases. Panel B shows the backbones of the heterodimeric enzyme from D. fructosovorans (pdb 1YQW). Panel C shows the H cluster of FeFe hydrogenases (a star marks the H_2 binding site), and panel D shows the backbone of the enzyme from *Chlamydomonas* reinhardtii (pdf 3LX4).

 The Fe ion binds carbonyl and cyanide ligands which also occur at the active site of FeFe hydrogenases, the "H cluster", shown in fig 1C.

 The NiFe hydrogenases that have been crystallized so far all look like the 10 heterodimeric enzyme from D. fructosovorans, whose structure is shown in fig. 1B. A chain of three FeS clusters (whose exact nature varies) wires the active site to the soluble redox partner or the electrode. The FeFe hydrogenase from ¹³ C. reinhardtii whose structure is shown in fig 1D has no other cofactor than the surface-exposed H cluster, but other FeFe hydrogenases embed accessory FeS clusters for long range electron transfer (ET).

 Evidence for direct electron transfer between electrodes and hydrogenases goes all the way back to the 1980's [10, 35, 71], and the research in this field has expanded enormously over the last ten years, driven by the objective of using hydrogenases in solar-fuels devices. When direct electrochemistry is used in the context of enzyme kinetics, it provides unequalled redox control, time resolution and accuracy of the activity measurement, together with 22 flexibility in terms of transient exposures to inhibitors and/or substrate. If such kinetic information is combined with that obtained from other methods, the potential for gaining original information becomes enormous.

Electrodes for direct electron transfer

 Hydrogenases are easily wired to electrodes because they all have at least one surface-exposed redox center, either the active site (e.g. fig 1D) or the final redox relay (e.g. fig 1B), which allows fast electron entry/exit. (This is unlike other redox enzymes such as glucose oxidase where the active site is buried and isolated in the protein matrix.) Many hydrogenases oxidize and produce hydrogen at very high rates (in excess of thousands per second, table 4 in ref [75]), and therefore a catalytic current may be detected even if the amount of enzyme that is attached to the electrode is very low. This explains the diversity of electrode materials that have proven useful in this context, listed in table 1 (see also refs [76–78] for reviews). Finely designed architectures for embedding membrane-bound hydrogenases have also been developed [79–81].

Heterogeneous reconstitution

 In the natural biosynthetic pathway of FeFe hydrogenase, a 2Fe frag- ment of the H cluster is delivered to the "apo" form of the enzyme. The ⁴¹ recent discovery that a *synthetic* 2Fe fragment can be incorporated into apo- hydrogenase has revolutionized hydrogenase research [82, 83]. The use of direct electrochemistry to probe the kinetics of such artificial maturation of the enzyme is one of the most recent and exciting developments: the apo- enzyme can be adsorbed onto an electrode and can incorporate the 2Fe syn- thetic subcluster when the latter is added to the solution. The reconstitution $\frac{47}{47}$ of a holo-active enzyme results in an increase in H₂-oxidation current which reports on the rate of reconstitution [84]. (This is reminiscent of the recent evidence by Limoges and coworkers that the PQQ cofactor of glucose dehy- drogenase can bind the apo-enzyme anchored on an electrode surface [85].) Armstrong and coworkers could design potential-steps PFV experiments to detect the formation of an intermediate that is probably relevant to the final stage of biological H cluster maturation.

Electrochemical studies of exotic hydrogenases

 The electrochemical literature does not accurately reflect the biodiversity of hydrogenases. Indeed, most of the hydrogenases that have been studied in electrochemistry are very similar to each other. They are relatively small (60–100 kDa), and consist of a small number of proteins subunits, most often one or two. In contrast, some hydrogenases are part of very large enzymatic systems (e.g. 18 subunits and 600 kDa in the formate hydrogene ⁶¹ lyase complex of *Thermococcus*). These complex hydrogenases use the same active sites as those shown in fig 1, but their protein sequences, quaternary structure and cofactor content make them unique.

 Recent electrochemical studies of hydrogenases whose structures are out of the ordinary [19, 22, 23, 73] have revealed unprecedented properties. For example, the NiFe hydrogenase of the hyperthermophilic bacterium P. furio- σ sus remains active upon exposure to O_2 at 80°C, and its mechanism of O_2 68 tolerance is uncommon [23]. The FeFe hydrogenase of A. woodii is part of a 69 large complex which reduces $CO₂$ to formate; it has high affinity for $H₂$ and is reversibly inhibited by CO under all redox conditions [73], unlike other FeFe hydrogenases, for which the inhibition is only partly reversible under reducing conditions [70].

 Together with site-directed mutagenesis studies, the characterization of these non-standard hydrogenases demonstrates that the catalytic properties depend on the environment of the active site, but also on structural elements that are remote from the conserved active site.

The catalytic bias

 Studies of hydrogenases have recently encouraged discussions on an im- portant and often neglected aspect of enzyme catalysis: the question of what makes a particular enzyme a better catalyst in one direction of the reaction than in the other [91]. Answering this question is very important in the con- text of solar fuels research, for the rational design of either synthetic catalysts $83 \quad [92]$ or bacterial strains [93] that either produce or oxidize H₂.

84 One defines the catalytic bias of a hydrogenase as the ratio of the H₂ δ ⁸⁵ oxidation and H₂ production rates, which have to be measured under two different sets of experimental conditions. In PFV experiments, sweeping the potential makes it possible to probe the enzymatic response on either side of the equilibrium potential (OCP) in a single experiment. Any predisposition

Figure 2: Cyclic voltammograms obtained with hydrogenases undergoing direct ET with a rotating electrode, recorded under an atmosphere of either N_2 (gray lines) or H_2 (black lines). The oxydation current is counted as positive. Arrows mark the directions of the potential sweeps. The dotted lines are blanks. A: H_2 oxidation by A. vinosum NiFe hydrogenase (the fast scan rate used here prevents oxidative inactivation) [86]. B: H_2 oxidation and evolution by C. acetobutilicum FeFe hydrogenase [53]. C: high potential inactivation of A. aeolicus NiFe hydrogenase [25]. D: high potential inactivation of a sitedirected mutant of C. reinhardtii FeFe hydrogenase [56]. E: H_2 oxidation and evolution by D. fructosovorans NiFe hydrogenase at pH 4 or 6 (signals at higher or lower potential, respectively), all other things being equal [87]. F: low potential reversible inactivation of C. reinhardtii FeFe hydrogenase at pH 7 [88]. In all cases here, the absence of current plateau results from the distribution of interfacial ET rate constants [86, 89, 90].

89 for oxidizing or producing H_2 is thus qualitatively observed as a larger current ⁹⁰ in one particular direction (fig 2) [94]. (Of course, the rate of H_2 evolution is likely to be larger under more acidic conditions because the proton is ⁹² the substrate [87, 95], and H_2 production is also faster when H_2 is removed 93 [95, 96]; see fig 2E.)

 A standardized method for measuring the bias (either in solution assays or on an electrode) is lacking, but the comparison between various results such as those in fig. 2 suggested that NiFeSe [43, 44, 100] and FeFe hy- σ drogenases are better H₂-production catalysts than their NiFe counterparts γ ₉₈ (see however ref [22]). The O₂-resistant NiFe hydrogenases are essentially 99 unidirectional H₂-oxidation enzymes whereas the standard $(O_2$ -sensitive) en- zymes are bidirectional catalysts [101], and certain mutations that strongly increase the O₂-tolerance of standard NiFe hydrogenase also selectively slow $_{102}$ H₂-evolution [12, 102–104]. Other mutations of amino acids that are remote from the active site also alter the bias of NiFe hydrogenases [105–107].

 There is much interest in understanding the bias in relation to the shape of the catalytic signal and the properties of the catalytic intermediates. The ratio of oxidative and reductive currents is inevitably related to the difference 107 between the two-electron "catalytic potential" E_{cat} and the OCP by eq. 1 in box 1, but various models give distinct answers to the question of what defines E_{cat} . Emphasis was often on the role of the redox relays [89, 97, 107] (Box 1), but there is one clear example from our group where the bias is defined $_{111}$ by the H₂ diffusion rates along the gas channel [104].

 The observed rate of catalysis in either direction is also affected by any redox-driven inactivation of the enzyme, unmistakably detected as a hystere- sis in cyclic voltammetry at a rotating disc electrode (RDE) [90]. NiFe hy- drogenases reversibly inactivate under oxidative conditions [12, 25, 43, 108], whereas FeFe hydrogenases inactivate in a complex manner both at high [56, 68, 73] and low potential [84, 88] (see e.g. figs 2C, D and F, respec-tively).

Combining electrochemistry and theoretical chemistry

 (Electro)kinetic measurements alone most often cannot give the molec- ular mechanism of a reaction whose rate is being measured. An approach that has recently gained popularity is to combine electrochemistry and the- oretical chemistry [109]. In this context, a seminal PFV paper addressed the mechanism of electron/proton transfer to a buried FeS cluster in a small

 protein [110]: electrochemical experiments identified the "kinetic mechanism" (sequential, rather than concerted), and the results of molecular dynamics (MD) simulations and site-directed mutagenesis (SDM) experiments could be used to describe the molecular details of the reaction.

129 More recently, the approach that combines PFV and theoretical calcu- lations (DFT and/or MD) proved powerful to decipher the mechanism of 131 inhibition of FeFe hydrogenases by formaldehyde [111–113], CO [57, 70], O_2 [57], or under anaerobic oxidative conditions [56]. These studies concern the active site mechanism, but also the diffusion along gas channels and binding at the active site of small molecules: the rates of these reactions can be mea- sured using electrochemistry and sometimes calculated — this was achieved with both NiFe [55, 109, 114, 115] and FeFe hydrogenases [54, 57].

Direct spectroelectrochemistry

 The vibrations of the carbonyl and cyanide ligands at the active sites 139 of hydrogenases result from IR absorptions in the $1800-2100$ cm⁻¹ region, which is devoid of other absorption bands, allowing selective detection of the active site. Spectroelectrochemical titrations followed by FTIR spectroscopy have identified various states of the active sites, some of which are probably catalytic intermediates. The caveat of this approach is that a system that reduces protons can only reach equilibrium under a certain pressure of hy-145 drogen that becomes much too large at potentials below $E^{0'} = -0.06 \times pH$. 146 For instance, equilibration at -450 mV at pH 7 requires a H₂ pressure of 10 bars, much too high for a spectroelectrochemical cell to withstand. Re- cently, various groups have gone beyond equilibrium titrations by combining standard catalytic protein film voltammetry with the simultaneous detection of the active site spectroscopic signatures of NiFe hydrogenases; this made it possible to correlate the features of the catalytic response and specific states of the active site, as illustrated below.

 Surface-enhanced infra-red absorption spectroscopy, with NiFe hydroge- nases adsorbed onto modified gold electrodes, has been used to relate the appearance of the NiB active site signature to the disappearance of activity at high potentials [116, 117].

 A significant breakthrough came from the Vincent group in Oxford, who combined an attenuated total reflection cell with a volumic carbon black elec- trode [118], giving electrochemical signals strongly resembling those obtained with a "flat" PGE electrode, with enough sensitivity to characterize species that had previously escaped detection. Vincent and coworkers showed that the Ni-L state, long thought to be an artifact of illumination at cryogenic temperatures, is one of the possible intermediates of the oxidation of Ni-C [32], with which it is in acid-base equilibrium [119]. They could also detect for the first time the presence of Ni-R, the most reduced state in the catalytic 166 cycle, in R. eutropha regulatory hydrogenase $[120]$.

Direct photoelectrochemistry

 The active sites of hydrogenases bearing Fe-carbonyl bonds, it is no sur- prise that these enzymes are sensitive to light in the UV-vis range. It has long been know that exposure to white light reactivates the FeFe hydrogenase bound to extrinsic CO, but until recently the photochemistry of hydrogenases was much less studied than that of hydrogenase synthetic mimics.

 In spectroscopic investigations, information on the electronic structure of the active site is obtained from the knowledge of which wavelengths are absorbed by the cluster; in contrast, with the enzyme wired to an electrode, one can detect light absorption by monitoring wavelength-dependent light- induced changes in turnover rate. This is possible even when the protein or chromophores hide the weak absorption that triggers the change in activity. By monitoring changes in turnover rate, one is certain to focus on light absorption events that impact the catalytic intermediates.

 Regarding FeFe hydrogenases, the first direct photoelectrochemistry ex- periments focused on the inhibited form of the active site and the enhance- ment of the rate of release of extrinsic CO by white light [68]. More recently, we could observe that this enhancement only occurs in the blue part of the visible spectrum, and it is proportional to light power; the effect is small because the H cluster has very low absorption in the visible range, but the quantitative analysis of the electrochemical data demonstrates that every photon that is absorbed by the inhibited H cluster induces CO release [72].

 It had also been reported that day-light destroys the H cluster of D. desulfuricans FeFe hydrogenase [121], which certainly raises questions as to 191 whether FeFe hydrogenases can be coupled to photosensitizers for solar H_2 192 production. White light photo-electrochemistry experiments with $D.$ desul- furicans FeFe hydrogenase gave a less dramatic picture [122]. In the case of 194 the enzymes from C. acetobutylicum and C. reinhardtii, recent results demon-strate that photoinhibition only occurs upon UV irradiation, which triggers

 the release of an intrinsic CO ligand followed by irreversible isomerisation of the H cluster [123].

 Lojou and coworkers also discovered that irradiation at 405 nm activates 199 the O₂-tolerant NiFe hydrogenase from A. aeolicus under turnover condi- tions [124]. The mechanism and action spectrum of this unprecedented reac- tion and the implications regarding the nature of the inactive states of NiFe hydrogenases must be explored.

Conclusion

 The abundant literature in the field of hydrogenase electrochemistry re- flects the ease with which catalytic currents are obtained, the variety of the mechanistic questions that these studies can answer, the large number of applications which rely on wiring hydrogenases to electrodes, nanoparticles or photosensitizers [77, 125–128] and, of course, the creativity and combined talent of many biochemists and electrochemists. The implications of these results are wide, from the understanding of fundamental concepts in electro- catalysis to implications for the design of solar-fuel catalysts. We hope that they will also prove inspirational in other, related fields of research.

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Table 1: The hydrogenases that have been directly connected to electrodes, and the natures of the electrodes used in these studies. bSi: nanoporous black silicon CC/CB: carbon black on carbon cloth, CDC: carbodiimide coupling, CF: carbon felt, NC: nanocrystals, NP: nano-particles, GC: glassy carbon, GC/KB: Ketjen black-modified GC, CNT: carbon nanotubes, CNF: carbon nanofibers, MPDB: electropolymerized pyrrol, PGE: pyrolytic graphite edge [74], PGB: basal plane of PG, PGP: PG particles, QD: quantum dots, SAM: self assembled monolayers,

If the steady-state catalytic wave shape is sigmoidal, as observed in the case of hydrogenases, a simple equation relates the two-electron "catalytic potential" E_{cat} , the open circuit potential (Nernst potential of the H^+/H_2 couple), and the catalytic bias:

$$
\frac{i_{\text{ox}}}{i_{\text{red}}} = \exp\left[\frac{2F}{RT}\left(E_{\text{cat}} - E_{\text{OCP}}\right)\right]
$$
\n(1)

The value $n = 2$ comes from the stoichiometry of the redox reaction, and eq. 1 results from the condition that $i = 0$ at the OCP.

For a two-electron redox reaction, if the catalytic signal is a two-electron sigmoid (left panel), E_{cat} in eq. 1 is the mid-wave potential; if the wave consists of two one-electron waves (right panel), E_{cat} is the average value of the two mid-wave potentials. In intermediate cases or if the signal is broadened by slow interfacial ET, E_{cat} must be determined by fitting [90]. Verifying eq 1 in a particular case does not give an explanation for the bias. What matters is the reason E_{cat} takes a particular value. According to models that only take into account one relay (no active site, no redox chain, no kinetics of intramolecular ET), E_{cat} can only equate the potential of the unique redox center in the model, the relay [97]; this was taken as evidence that the difference between the potential of the entry/exit relay and the OCP defines the catalytic bias [98]. In contrast, according to models that take into account reversible catalysis, one-electron mediation, and two-electron active-site chemistry, the *two-electron* potential E_{cat} cannot strictly equate the *one-electron* potential of any of the redox relays [89]; E_{cat} tends to the potential of the active site if intramolecular ET is very fast, but its value is influenced by the kinetics and thermodynamics of intramolecular ET, and the kinetics of all steps in the catalytic cycle that are coupled to active site redox chemistry [89, 90, 99].

Box 1: The relation between catalytic overpotential and the catalytic bias, when the catalytic signal is sigmoidal.

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