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New perspectives in hydrogenase direct electrochemistry

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Abstract

Electrochemical studies of hydrogenases, the biological catalysts of H₂ 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

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

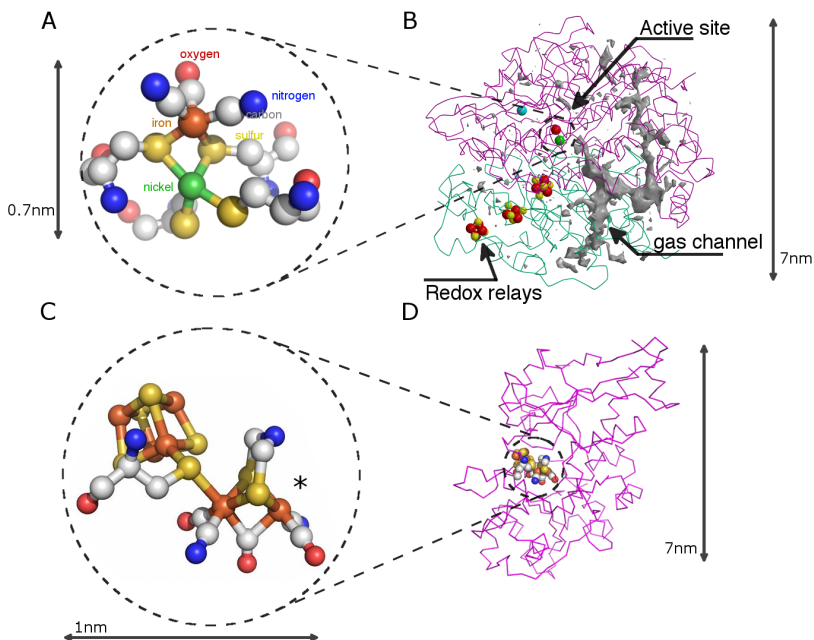


Figure 1: The X-ray structures of two hydrogenases. Panel A shows the active site of NiFe hydrogenases. Panel B shows the backbones of the heterodimeric enzyme from *D. fructosovorans* (pdb 1YQW). Panel C shows the H cluster of FeFe hydrogenases (a star marks the H₂ binding site), and panel D shows the backbone of the enzyme from *Chlamydomonas reinhardtii* (pdf 3LX4).

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

9 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
 11 1B. A chain of three FeS clusters (whose exact nature varies) wires the active
 12 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
 14 the surface-exposed H cluster, but other FeFe hydrogenases embed accessory
 15 FeS clusters for long range electron transfer (ET).

16 Evidence for direct electron transfer between electrodes and hydrogenases
 17 goes all the way back to the 1980’s [10, 35, 71], and the research in this field
 18 has expanded enormously over the last ten years, driven by the objective of
 19 using hydrogenases in solar-fuels devices. When direct electrochemistry is

20 used in the context of enzyme kinetics, it provides unequalled redox control,
21 time resolution and accuracy of the activity measurement, together with
22 flexibility in terms of transient exposures to inhibitors and/or substrate. If
23 such kinetic information is combined with that obtained from other methods,
24 the potential for gaining original information becomes enormous.

25 **Electrodes for direct electron transfer**

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

38 **Heterogeneous reconstitution**

39 In the natural biosynthetic pathway of FeFe hydrogenase, a 2Fe frag-
40 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-
42 hydrogenase has revolutionized hydrogenase research [82, 83]. The use of
43 direct electrochemistry to probe the kinetics of such artificial maturation of
44 the enzyme is one of the most recent and exciting developments: the apo-
45 enzyme can be adsorbed onto an electrode and can incorporate the 2Fe syn-
46 thetic subcluster when the latter is added to the solution. The reconstitution
47 of a holo-active enzyme results in an increase in H₂-oxidation current which
48 reports on the rate of reconstitution [84]. (This is reminiscent of the recent
49 evidence by Limoges and coworkers that the PQQ cofactor of glucose dehy-
50 drogenase can bind the apo-enzyme anchored on an electrode surface [85].)
51 Armstrong and coworkers could design potential-steps PFV experiments to
52 detect the formation of an intermediate that is probably relevant to the final
53 stage of biological H cluster maturation.

54 **Electrochemical studies of exotic hydrogenases**

55 The electrochemical literature does not accurately reflect the biodiversity
56 of hydrogenases. Indeed, most of the hydrogenases that have been studied
57 in electrochemistry are very similar to each other. They are relatively small
58 (60–100 kDa), and consist of a small number of proteins subunits, most
59 often one or two. In contrast, some hydrogenases are part of very large
60 enzymatic systems (e.g. 18 subunits and 600 kDa in the formate hydrogenase
61 lyase complex of *Thermococcus*). These complex hydrogenases use the same
62 active sites as those shown in fig 1, but their protein sequences, quaternary
63 structure and cofactor content make them unique.

64 Recent electrochemical studies of hydrogenases whose structures are out
65 of the ordinary [19, 22, 23, 73] have revealed unprecedented properties. For
66 example, the NiFe hydrogenase of the hyperthermophilic bacterium *P. furio-*
67 *sus* remains active upon exposure to O₂ at 80°C, and its mechanism of O₂
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
70 is reversibly inhibited by CO under all redox conditions [73], unlike other
71 FeFe hydrogenases, for which the inhibition is only partly reversible under
72 reducing conditions [70].

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

77 **The catalytic bias**

78 Studies of hydrogenases have recently encouraged discussions on an im-
79 portant and often neglected aspect of enzyme catalysis: the question of what
80 makes a particular enzyme a better catalyst in one direction of the reaction
81 than in the other [91]. Answering this question is very important in the con-
82 text of solar fuels research, for the rational design of either synthetic catalysts
83 [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
85 oxidation and H₂ production rates, which have to be measured under two
86 different sets of experimental conditions. In PFV experiments, sweeping the
87 potential makes it possible to probe the enzymatic response on either side of
88 the equilibrium potential (OCP) in a single experiment. Any predisposition

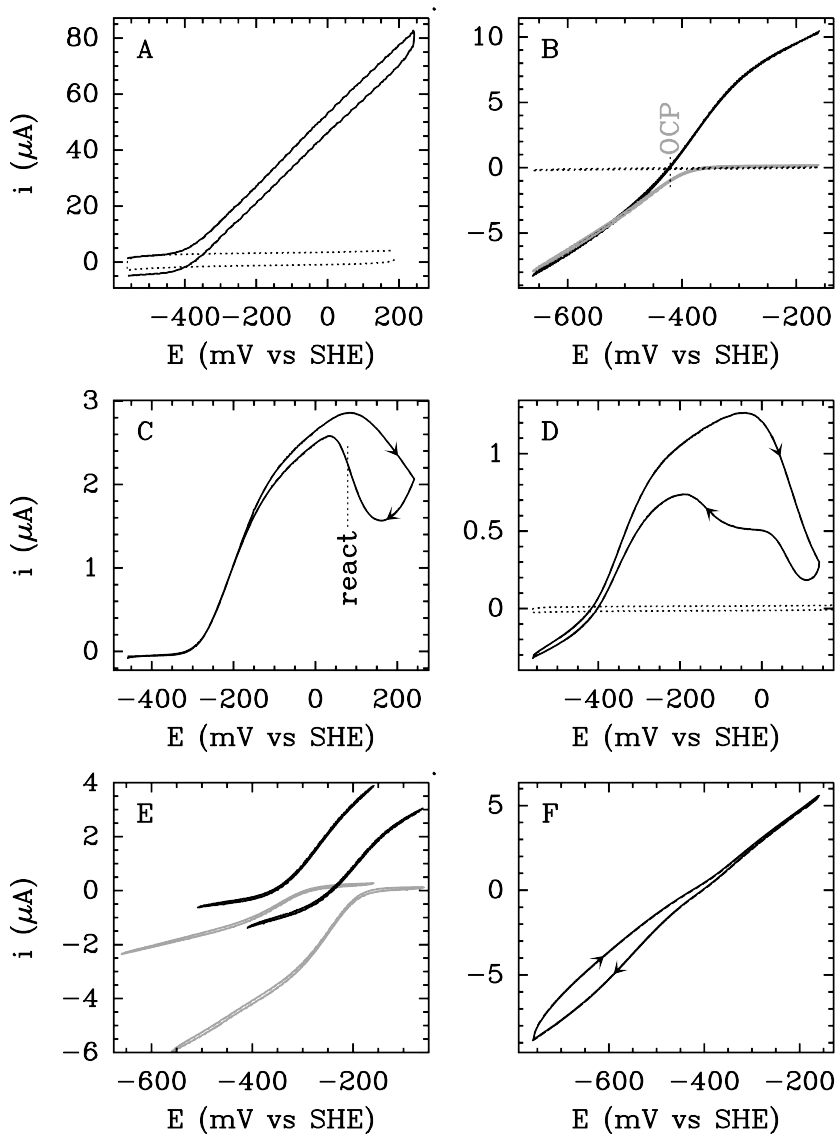


Figure 2: Cyclic voltammograms obtained with hydrogenases undergoing direct ET with a rotating electrode, recorded under an atmosphere of either N_2 (gray lines) or H_2 (black lines). The oxidation current is counted as positive. Arrows mark the directions of the potential sweeps. The dotted lines are 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. acetobutylicum* FeFe hydrogenase [53]. C: high potential inactivation of *A. aeolicus* NiFe hydrogenase [25]. D: high potential inactivation of a site-directed 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
91 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.)

94 A standardized method for measuring the bias (either in solution assays
95 or on an electrode) is lacking, but the comparison between various results
96 such as those in fig. 2 suggested that NiFeSe [43, 44, 100] and FeFe hy-
97 drogenases are better H_2 -production catalysts than their NiFe counterparts
98 (see however ref [22]). The O_2 -resistant NiFe hydrogenases are essentially
99 unidirectional H_2 -oxidation enzymes whereas the standard (O_2 -sensitive) en-
100 zymes are bidirectional catalysts [101], and certain mutations that strongly
101 increase the O_2 -tolerance of standard NiFe hydrogenase also selectively slow
102 H_2 -evolution [12, 102–104]. Other mutations of amino acids that are remote
103 from the active site also alter the bias of NiFe hydrogenases [105–107].

104 There is much interest in understanding the bias in relation to the shape
105 of the catalytic signal and the properties of the catalytic intermediates. The
106 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
108 box 1, but various models give distinct answers to the question of what defines
109 E_{cat} . Emphasis was often on the role of the redox relays [89, 97, 107] (Box
110 1), but there is one clear example from our group where the bias is defined
111 by the H_2 diffusion rates along the gas channel [104].

112 The observed rate of catalysis in either direction is also affected by any
113 redox-driven inactivation of the enzyme, unmistakably detected as a hystere-
114 sis in cyclic voltammetry at a rotating disc electrode (RDE) [90]. NiFe hy-
115 drogenases reversibly inactivate under oxidative conditions [12, 25, 43, 108],
116 whereas FeFe hydrogenases inactivate in a complex manner both at high
117 [56, 68, 73] and low potential [84, 88] (see e.g. figs 2C, D and F, respec-
118 tively).

119 Combining electrochemistry and theoretical chemistry

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

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

129 More recently, the approach that combines PFV and theoretical calcu-
130 lations (DFT and/or MD) proved powerful to decipher the mechanism of
131 inhibition of FeFe hydrogenases by formaldehyde [111–113], CO [57, 70], O₂
132 [57], or under anaerobic oxidative conditions [56]. These studies concern the
133 active site mechanism, but also the diffusion along gas channels and binding
134 at the active site of small molecules: the rates of these reactions can be mea-
135 sured using electrochemistry and sometimes calculated — this was achieved
136 with both NiFe [55, 109, 114, 115] and FeFe hydrogenases [54, 57].

137 **Direct spectroelectrochemistry**

138 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,
140 which is devoid of other absorption bands, allowing selective detection of the
141 active site. Spectroelectrochemical titrations followed by FTIR spectroscopy
142 have identified various states of the active sites, some of which are probably
143 catalytic intermediates. The caveat of this approach is that a system that
144 reduces protons can only reach equilibrium under a certain pressure of hy-
145 drogen that becomes much too large at potentials below $E^{0'} = -0.06 \times \text{pH}$.
146 For instance, equilibration at -450 mV at pH 7 requires a H₂ pressure of
147 10 bars, much too high for a spectroelectrochemical cell to withstand. Re-
148 cently, various groups have gone beyond equilibrium titrations by combining
149 standard catalytic protein film voltammetry with the simultaneous detection
150 of the active site spectroscopic signatures of NiFe hydrogenases; this made it
151 possible to correlate the features of the catalytic response and specific states
152 of the active site, as illustrated below.

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

157 A significant breakthrough came from the Vincent group in Oxford, who
158 combined an attenuated total reflection cell with a volumic carbon black elec-
159 trode [118], giving electrochemical signals strongly resembling those obtained
160 with a “flat” PGE electrode, with enough sensitivity to characterize species

161 that had previously escaped detection. Vincent and coworkers showed that
162 the Ni-L state, long thought to be an artifact of illumination at cryogenic
163 temperatures, is one of the possible intermediates of the oxidation of Ni-C
164 [32], with which it is in acid-base equilibrium [119]. They could also detect
165 for the first time the presence of Ni-R, the most reduced state in the catalytic
166 cycle, in *R. eutropha* regulatory hydrogenase [120].

167 **Direct photoelectrochemistry**

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

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

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

189 It had also been reported that day-light destroys the H cluster of *D.*
190 *desulfuricans* FeFe hydrogenase [121], which certainly raises questions as to
191 whether FeFe hydrogenases can be coupled to photosensitizers for solar H₂
192 production. White light photo-electrochemistry experiments with *D. desul-*
193 *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-
195 strate that photoinhibition only occurs upon UV irradiation, which triggers

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

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

203 **Conclusion**

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

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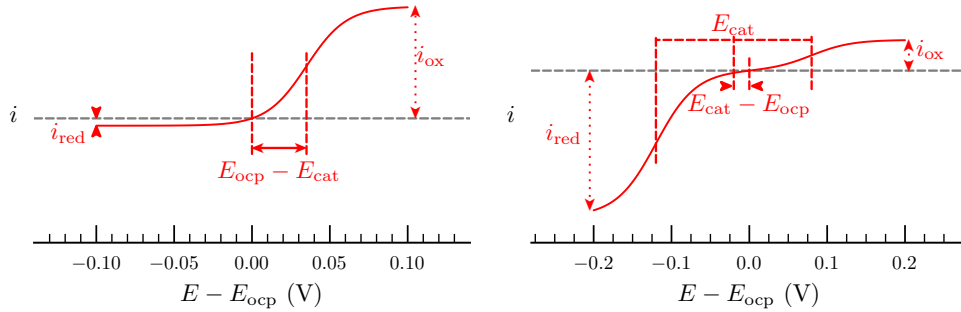
Type	Source	Attachement methods
NiFe	<i>A. vinosum</i>	PGE/polymyxin [1, 2], Au/polymyxin [3, 4], PGP [5]
	<i>D. gigas</i>	Au/SAM/CDC [6], GC [7], PGE/CDC [8], CNT/CDC [9]
	<i>D. vulgaris</i> Hildenborough	PGE/polylysine [10], basal PGE [11]
	<i>D. fructosovorans</i>	PGE [12], PGE/CDC [13], CNT[14]
	<i>E. coli</i> (Hyd-1)	PGE [15], pyrenyl carbon nanostructures [16]
	<i>E. coli</i> (Hyd-2)	PGE [17], PGP [18]
	<i>E. coli</i> (Hyd-3)	PGE/CNT/polymixin [19]
	<i>E. coli</i> membrane fractions	TiO ₂ [20]
	<i>Citrobacter</i> sp. S-77	CC/CB [21]
	<i>Synechocystis</i> sp. PCC 6803	PGE [22]
	<i>P. furiosus</i> (hydrogenase I)	PGE [23]
	<i>A. aeolicus</i>	PGB [24], PGE [25], Au/SAM [26], CNT [27], CNF [28], SWCNT/CDC[29]
	<i>R. eutropha</i> (MbH)	PGE(FAA), AU/SAM [30, 31]
	<i>R. eutropha</i> (RH)	CB [32]
	<i>R. metallidurans</i> CH34 (Rm)	PGE [33]
	<i>R. eutropha</i> (SH)	PGE [34]
	<i>T. roseopersicina</i>	CB [35], CNT [36], CNF [37], CNF/MPDB [38]
<i>H. marinus</i>	Au/CDC [39], GC & Gc/KB [40]	
NiFeSe	<i>D. vulgaris</i> Hildenborough	Au/SAM/CDC [6, 41, 42], PGE [43]
	<i>D. baculatum</i>	PGE [44], TiO ₂ NP [45], mesoporous TiO ₂ [46], SiTiO ₂ [47]
FeFe	<i>C. acetobutylicum</i>	TiO ₂ [48], CF [49], Au/SAM [50], CNT [51, 52], PGE [53–57], PGE/CDC [58–60], CdS [61–63], CdTe NC [64], CdTe QD [65], bSi [66]
	<i>C. perfringens</i>	TiO ₂ [67]
	<i>D. desulfuricans</i>	PGE [54, 55, 68], PGE/CDC [59]
	<i>C. reinhardtii</i>	Au/SAM [69], PGE [54, 59, 70], PGE/CDC [58]
	<i>M. elsdenii</i>	GC [71], PGE[72]
	<i>A. woodii</i>	PGE [73]

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:

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

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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