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Electrochemical characterization of a complex FeFe hydrogenase, the electron-bifurcating Hnd from *Desulfovibrio fructosovorans*

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2 ABSTRACT

3 Hnd, an FeFe hydrogenase from *Desulfovibrio fructosovorans*, is a tetrameric enzyme that can
4 perform flavin-based electron bifurcation. It couples the oxidation of H₂ to both the exergonic
5 reduction of NAD⁺ and the endergonic reduction of a ferredoxin. We previously showed that Hnd
6 retains activity even when purified aerobically unlike other electron-bifurcating hydrogenases.
7 In this study, we describe the purification of the enzyme under O₂-free atmosphere and its
8 biochemical and electrochemical characterization. Despite its complexity due to its multimeric
9 composition, Hnd can catalytically and directly exchange electrons with an electrode. We
10 characterized the catalytic and inhibition properties of this electron-bifurcating hydrogenase
11 using protein film electrochemistry of Hnd by purifying Hnd aerobically or anaerobically, then
12 comparing the electrochemical properties of the enzyme purified under the two conditions *via*
13 protein film electrochemistry. Hydrogenases are usually inactivated under oxidizing conditions in
14 the absence of dioxygen and can then be reactivated, to some extent, under reducing conditions.
15 We demonstrate that kinetics of this high potential inactivation/reactivation for Hnd show original
16 properties: it depends on the enzyme purification conditions and varies with time, suggesting the
17 coexistence and the interconversion of two forms of the enzyme. We also show that Hnd catalytic
18 properties (K_m for H₂, diffusion and reaction at the active site of CO and O₂) are comparable to
19 those of standard hydrogenases (those which cannot catalyze electron bifurcation). These results
20 suggest that the presence of the additional subunits, needed for electron bifurcation, changes
21 neither the catalytic behavior at the active site, nor the gas diffusion kinetics but induces unusual
22 rates of high potential inactivation/reactivation.

23 **Keywords:** direct electrochemistry, FeFe hydrogenase, electron bifurcation, *Desulfovibrio fructosovorans*, inactivation

1 ABBREVIATIONS:

24 FMN: Flavin mononucleotide, NAD: Nicotinamide adenine dinucleotide, NADP: Nicotinamide adenine
25 dinucleotide phosphate, *bis*PGD: *bis* pyranopterin guanosine dinucleotide, Fd: ferredoxin, SHE: standard
26 hydrogen electrode

2 INTRODUCTION

2.1 Electron-bifurcating enzymes

Oxidoreductase enzymes usually catalyze electron transfer between one electron donor and one electron acceptor. Electron-bifurcating enzymes are part of the oxidoreductase family but catalyze the reaction between two electron donors and one electron acceptor or one electron donor and two electron acceptors. More importantly, the reactions with the two electron donors or the two electron acceptors are thermodynamically coupled, one of the reactions being exergonic and the other endergonic. The global reaction being exergonic, the energetic coupling enables an endergonic reaction to occur (Baymann *et al.*, 2018). The first electron-bifurcating enzyme characterized was the cytochrome *bc₁* complex in which the electron-bifurcating site is a quinone (Mitchell, 1975). More recently, electron bifurcating enzymes were described in which the electron-bifurcation site is a flavin (Herrmann *et al.*, 2008). The common feature of quinones and flavins is their two redox transitions, making them 2-electron centers. A variety of enzymes were described to use the electron bifurcation mechanism for catalysis: electron-transferring flavoprotein (Etf), heterodisulfide reductase/hydrogenase (Hdr-Mvh), NADH-dependent ferredoxin:NADP⁺ oxidoreductase (Nfn) and NADH-dependent FeFe-hydrogenase to name but a few (Peters *et al.*, 2016; Buckel and Thauer, 2018). Electron bifurcation is a mechanism that is emerging as essential for the bioenergetic of many organisms, but this mechanism is still poorly understood, in part due to the low number of model enzymes characterized to date.

2.2 Electron-bifurcating hydrogenases

NADH-dependent electron-bifurcating hydrogenases are multimeric (tri- or tetrameric) enzymes and are classified A3 according to the hydrogenase classification proposed by Greening *et al.* (Søndergaard *et al.*, 2016; Greening *et al.*, 2016). They are all of FeFe-type and consist of at least one subunit harboring the catalytic H-cluster (the hydrogenase active site, which consists of a [4Fe4S] cluster bound *via* a cysteine to a 2Fe subcluster), a [2Fe2S]-cluster containing subunit and a subunit that contains a flavin (usually FMN) and FeS clusters as well as an NADH binding site. They catalyze the oxidation of H₂ coupled to the reduction of both NAD⁺ and a ferredoxin with a bifurcation mechanism and/or the reduction of proton coupled to oxidation of NADH and a ferredoxin with a confurcation mechanism. So far, electron-bifurcating hydrogenases from five anaerobic bacteria have been purified and characterized (Schuchmann and Mueller, 2012; Wang *et al.*, 2013; Zheng *et al.*, 2014), including the electron-bifurcating hydrogenase HydABC from *Thermotoga maritima* (Schut and Adams, 2009) and HndABCD from *Desulfovibrio fructosovorans* (Kpebe *et al.*, 2018). They have been tested for either electron bifurcation, or electron confurcation or for both (hydrogenase from *Moorella thermoacetica* (Wang *et al.*, 2013)). However, no 3D-structure of an electron bifurcating hydrogenase is available, and the electron pathway and the electron bifurcation site in these enzymes are still controversial subjects. Further characterization of electron bifurcating hydrogenases that could be models of this class of enzyme, will increase the understanding of the overall mechanism of electron bifurcation.

2.3 Electrochemistry of FeFe hydrogenases

Electrochemical techniques to study hydrogenases are developed as a complement to biochemical and spectroscopic techniques (Pershad *et al.*, 1999). The first electrochemical characterization of an FeFe hydrogenase was published on the HydA hydrogenase from *Megasphaera elsdenii* (Butt *et al.*, 1997; Caserta *et al.*, 2018). Since then, FeFe hydrogenases from several organisms have been studied using protein film voltammetry to determine their catalytic properties: HydAB from *Desulfovibrio desulfuricans*

68 (Parkin et al., 2006; Vincent et al., 2005; Goldet et al., 2009; Rodríguez-Maciá et al., 2018), HydA from
69 *Clostridium acetobutylicum* (Baffert et al., 2008; Goldet et al., 2009; Baffert et al., 2011, 2012; Kubas
70 et al., 2017; Orain et al., 2015), HydA1 from *Chlamydomonas reinhardtii* (Stripp et al., 2009; Goldet et al.,
71 2009; Baffert et al., 2011; Knörzer et al., 2012; Fourmond et al., 2014; Hajj et al., 2014; Kubas et al., 2017;
72 Orain et al., 2015), CpI, CpII and CpIII from *Clostridium pasteurianum* (Artz et al., 2019), and HydA from
73 *Solobacterium moorei* (Land et al., 2019). All these FeFe hydrogenases are prototypical A1 monomeric or
74 dimeric enzymes (Søndergaard et al., 2016). Different aspects of the catalytic properties of these enzymes
75 are studied by electrochemical methods: affinity for the substrate (K_m), effect of pH on the catalytic
76 properties, kinetics of inhibition by small molecules (CO , O_2 , S_2^- , formaldehyde), kinetics of oxidative
77 and reductive inactivation, and catalytic bias. Only recently was a multimeric Hydrogen Dependent Carbon
78 Dioxide Reductase (HDCR) from *Acetobacterium woodii* characterized by electrochemistry (Ceccaldi
79 et al., 2017). This hydrogenase is classified A4. It consists of four subunits: the hydrogenase subunit
80 hosting the H-cluster, the formate dehydrogenase subunit hosting the Mo-bisPGD cofactor and two subunits
81 containing several FeS clusters. Electrochemical experiments similar to those developed for prototypic
82 hydrogenases were performed. All these experiments on the different hydrogenases and variants (mutations
83 of specific amino acids) give insight into the catalytic mechanism of FeFe hydrogenases and the molecular
84 determinants of the inactivations. The second and last multimeric hydrogenase characterized by direct
85 electrochemistry belongs to the electron-bifurcating enzyme family (classified A3), the hydrogenase
86 HydABC from *Thermotoga maritima* (Chongdar et al., 2020). The enzyme is heterologously produced in
87 *Escherichia coli* and artificially matured with a synthetic diiron cofactor. The isolated hydrogenase catalytic
88 subunit, as well as the complex HydABC, are adsorbed onto an electrode and cyclic voltammograms are
89 recorded at various pH, to show that the isolated hydrogenase subunit and the trimeric complex behave in a
90 similar fashion.

91 Here, we aim at determining if the catalytic and inhibition properties are influenced by the additional
92 subunits present in NADH-dependent electron-bifurcating hydrogenases or by the condition of purification.
93 We study the electron-bifurcating hydrogenase, HndABCD from *Desulfovibrio fructosovorans* (shortened
94 name Hnd), by biochemical and electrochemical methods. Hnd is homologously produced and purified
95 in a fully matured form (Kpebe et al., 2018). Because Hnd is still active when purified aerobically, we
96 determine how the purification conditions (aerobic vs anaerobic) influence the catalytic behavior of Hnd.

3 MATERIAL AND METHODS

97 3.1 Enzyme purification

98 The production and the purification of Hnd hydrogenase from *Desulfovibrio fructosovorans* under aerobic
99 conditions were previously described (Kpebe et al., 2018). The procedure was modified to maintain
100 anaerobic conditions: all steps were performed in a glove-box (Jacomex, $[\text{O}_2] \leq 2$ ppm) except the
101 ultra-centrifugation step, for which anaerobiosis is maintained in the tube due to an airtight plug. The
102 cell lysis was performed by sonication (10 cycles of 30 seconds) and the Strep-tagged hydrogenase was
103 purified on a StrepTactin- Superflow (IBA) column (20 mL). Purification was done according to the
104 manufacturer's instructions. For the comparison of the catalytic properties, the bacterial culture was split in
105 two, and purifications of the Hnd hydrogenase were performed in parallel under both anaerobic and aerobic
106 conditions.

107 3.2 Catalytic activity determination

108 All assays were performed at 30 °C and under anaerobic conditions.

109 3.2.1 H₂ oxidizing activity with methyl-viologen (MV)

110 H₂-oxidizing activity measurements were performed in anaerobic quartz cuvettes, under a pressure of
111 H₂ of 1 bar, in 800 μL of a reaction mixture containing 100 mM Tris-HCl pH 8.0, 2 mM dithiothreitol
112 (DTT), and 50 mM methyl-viologen (MV) (Sigma Aldrich) as an artificial electron acceptor. MV reduction
113 was monitored at 604 nm ($\epsilon = 13,600 \text{ M}^{-1} \cdot \text{cm}^{-1}$) using a UV-Vis spectrophotometer Lambda 25 (Perkin
114 Elmer), between 10 and 200 ng of purified Hnd were added to the mixture to start the reaction. One unit of
115 hydrogenase activity corresponds to the uptake of 1 μmol of H₂/min.

116 3.2.2 H₂-production activity with methyl-viologen (MV)

117 H₂-production assays were carried out using dithionite-reduced MV (50 mM of MV were reduced with
118 0.1 M sodium dithionite) as electron donor, in anaerobic 7 mL-serum bottles containing 1 mL of a reaction
119 mixture composed of 100 mM Tris-HCl pH 8.0. The gas phase was 100% N₂. H₂ production was measured
120 using gas chromatography (GC) as previously described (Avilan et al., 2018) and the reaction was started
121 by the addition of 0.3 to 1.1 μg of purified Hnd. One unit of hydrogenase activity corresponds to the
122 production of 1 μmol of H₂/min.

123 3.2.3 Electron-bifurcating activity for H₂ production

124 Electron-bifurcating (NAD⁺- and Fd-dependent) H₂-oxidizing activity was assayed as described
125 previously (Kpebe et al., 2018): in anaerobic quartz cuvettes, under 1 bar of H₂, in 800 μL-mixture
126 containing 100 mM Tris-HCl pH 8.0, 5 μM of FMN, 5 μM of FAD, and 3 mM NAD⁺ in the presence of 20
127 μM of purified FdxB ferredoxin from *D. fructosovorans*. NAD⁺- and FdxB-reduction were followed
128 simultaneously by recording a full spectrum every 30 s from 300 nm to 800 nm for 1 h, using a
129 Cary 60 (Varian) in a glovebox. NAD⁺- and FdxB-reduction rates were determined at 340 nm and
130 410 nm respectively using the QSoas software (Fourmond, 2016), an open source program available at
131 www.qsoas.org. The specific activity is given in μmol of NADH/min/mg. The absorption coefficients used
132 were: $\epsilon(\text{NADH}) = 6320 \text{ M}^{-1} \cdot \text{cm}^{-1}$, $\epsilon(\text{FdxB}_{\text{ox}}410\text{nm}) = 24,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and $\epsilon(\text{FdxB}_{\text{red}}410 \text{ nm}) = 12,000$
133 $\text{M}^{-1} \cdot \text{cm}^{-1}$ (Kpebe et al., 2018).

134 3.3 Electrochemical techniques

135 All electrochemical experiments were carried out with the electrochemical set-up and equipment described
136 in reference (Léger et al., 2004) in a glovebox (Jacomex) filled with N₂. 1 μL of Hnd enzyme solution was
137 mixed with 1 μL of DTT 1M and 8 μL of phosphate buffer pH 7. The final enzyme concentration was 2 to
138 7 μM. The enzyme was adsorbed (1 μL of the previous mix) onto a pyrolytic graphite edge electrode (PGE,
139 surface area $\approx 3 \text{ mm}^2$) previously polished with an aqueous alumina slurry (1 μm). The electrochemical
140 cell contained a pH 7 phosphate buffer and was continuously flushed with pure H₂ or with argon. The
141 temperature was regulated to the desired values by circulating water in the double jacket of the cell. For
142 measuring the rates of inhibition by O₂ or CO, a stock of a buffer saturated by 100% O₂ or 1% CO in
143 99% Argon was kept in a capped serum bottle and small aliquots of this solution were injected into the
144 electrochemical cell using gas-tight syringes. The concentrations of O₂ and CO were calculated using
145 the Henry's law constants: 1.25 mM (atm O₂)⁻¹ and 1 mM (atm CO)⁻¹. The change in H₂ solubility is
146 only about 12% between 10 °C and 30 °C (Wilhelm et al., 1977). This variation induces a difference in
147 the potential of H⁺/H₂ Nernst couple of 2 mV. With the surface area (around 5 mm²) of the electrode,
148 the maximum current (limitation by mass transport) is 100 μA. In the experiments of this study, the H₂
149 oxidation current (maximum 2 μA) is not limited by mass transport (or less than 2%) (Merrouch et al.,
150 2017). The data were analyzed using the QSoas software (Fourmond, 2016). The protein film loss was

151 included in the data analysis of Figure 4 according to reference(Fourmond et al., 2009). The effect of film
152 loss on cyclic voltammograms is shown in Figure S5.

4 RESULTS

153 4.1 Biochemical comparison between aerobically and anaerobically purified Hnd

154 We previously reported the biochemical characterization of the Hnd hydrogenase purified under aerobic
155 conditions (Kpebe et al., 2018). Here, we repeated the same characterization but with the enzyme purified
156 under anaerobic conditions. Table 1 summarizes the results obtained with the enzyme purified under the
157 two conditions. The conditions of purification do not have much influence on the properties of Hnd (Km
158 for methyl-viologen, optimal temperature and pH). If we compare the ratio of H₂ oxidation activity for the
159 two enzymes (anaerobically- and aerobically-purified Hnd) with methyl-viologen and with physiological
160 partners, the ratio is higher when considering the electron bifurcation activity. It should be noted that
161 the activities are lower than those previously reported (Kpebe et al., 2018) because experiments were
162 performed with enzyme purified from cells grown for a longer period (2 months, Covid19 lockdown period)
163 and at lower temperature (20 °C), and the enzyme samples were stored one week in liquid nitrogen before
164 electron-bifurcating activity measurements. We already observed that the enzyme activity decreases quickly
165 after purification (by a factor of 10) and then stabilizes (Kpebe et al., 2018). However, we checked that
166 only the specific activity changes upon storage and not the other biochemical and catalytic properties (See
167 Table S7).

168

169 4.2 Electrochemical characterization of Hnd

170 Hnd purified under the two conditions (aerobic and anaerobic) was characterized using electrochemical
171 methods developed in our laboratory (Del Barrio et al., 2018b; Sensi et al., 2017). As already observed for
172 the electron-bifurcating trimeric hydrogenase from *Thermotoga maritima* (Chongdar et al., 2020), despite
173 its multimeric form, the Hnd hydrogenase can transfer electrons directly to or from an electrode, without
174 the need for redox mediator. However, it not possible to speculate what is the first electron-relay within the
175 enzyme, *i.e.* what cofactor interacts with the electrode surface and whether it is unique (several entry points
176 could be possible) because structural information is not available for any electron-bifurcating hydrogenase.
177 Because experiments presented in this study were not performed in presence of NAD or ferredoxin, these
178 catalytic properties are more representative of a non-bifurcation reaction. We characterized Hnd purified
179 under aerobic and anaerobic conditions, and compared the catalytic and inactivation kinetic properties.

180 4.2.1 Oxidative inactivation and catalytic bias

181 4.2.1.1 Oxidative inactivation

182 Figure 1 shows catalytic cyclic voltammograms (current as the function of potential) of Hnd adsorbed
183 at a PGE electrode for three enzyme samples: aerobically-purified Hnd, anaerobically-purified Hnd, and
184 anaerobically-purified Hnd stored 1 day at 4 °C in a glove-box that contains about 10% dihydrogen. The
185 three voltammograms show a decrease in the catalytic current at high electrode potential, indicating an
186 oxidative inactivation that is reversible as shown on the reverse scan by the increase in current when the
187 potential is decreased. However, the reactivation is not complete, particularly for anaerobically purified
188 Hnd, as shown by the red voltammogram. The irreversible loss could be due to protein film desorption
189 and/or irreversible inactivation. We cannot discriminate between these two processes. It should be noted
190 that at this scan rate, for aerobically-purified Hnd (black line in Fig. 1), the decrease in current occurs at

191 around -0.15 V vs SHE. In the inset of Fig. 1, the cyclic voltammogram of the anaerobically-purified Hnd
192 (red line) shows two inactivation/reactivations (indicated by the arrows) at electrode potential around -0.35
193 V vs SHE and around -0.15 V vs SHE. After one day of storage of the anaerobically-purified enzyme, the
194 shape of the cyclic voltammogram is similar to that obtained with aerobically-purified Hnd (blue and black
195 lines in Fig. 1).

196 4.2.1.2 Catalytic bias

197 The catalytic bias (or catalytic preference (Fourmond et al., 2019; Del Barrio et al., 2018b; Sensi et al.,
198 2017)) is defined as the propensity of a catalyst to catalyze a reaction faster in one direction than in the
199 other. For the same positive and negative overpotential, the activity of the enzyme (*i.e.* the absolute value
200 of the current in electrochemical experiment) must be compared. The catalytic bias is mainly the function
201 of the rate limiting step of the catalyzed reaction. When inactivation happens, it influences the bias. As
202 shown in Figure 1 anaerobically-purified Hnd is biased toward hydrogen production because it inactivates
203 while oxidizing H₂. Because inactivation and catalytic rate constants vary with temperature, the bias can be
204 modulated by changing the temperature, as illustrated in Figure 2. While anaerobically-purified Hnd is
205 biased toward proton reduction at 30 °C, the opposite is true at 10 °C (See data in Table 2 for a chosen
206 overpotential of $\eta = \pm 100$ mV, the current of the forward scan was considered). The same behavior was
207 obtained for aerobically-purified and anaerobically-purified Hnd (Figure 2, Figure S1 and Table 2). The
208 same behavior was obtained for aerobically- and anaerobically-purified Hnd (Figure 2, Figure S1 and
209 Table 2). It should be noted that the potential range is not the same in Figure 1 (-0.56 to +0.04 V) and in
210 Figure 2 (-0.56 to -0.16 V); thus the second inactivation that happened around -0.15 V is not visible in
211 Figure 2.

212 4.2.2 Reductive inactivation

213 Hnd hydrogenase also inactivates at low electrode potential. We used the procedure described previously
214 to study this inactivation (Hajj et al., 2014): a chronoamperogram was recorded while the electrode potential
215 was changed in a 3-step-sequence (E₁-E₂-E₁). E₁ and E₂ values were chosen such as no activation nor
216 inactivation occurs at electrode-potential E₁, and such as detectable reductive inactivation takes place at
217 electrode potential E₂. After each step, a cyclic voltammogram (CV) was recorded. The CVs were started at
218 -0.5 V vs SHE and first scan to high potential. As shown in Figure 3, during the step at E = -760 mV vs SHE,
219 45% of the reduction current (*i.e.* enzyme activity) is lost, while the loss is only 30% after the third step
220 (percentage of the reduction current loss between the end of the first step and the third step) indicating that
221 inactivation is mostly irreversible. No reactivation was detected during this third step but fast inactivation
222 must take place during CV2. The shape of the cyclic voltammogram after low potential inactivation is
223 similar to the initial, thus the catalytic properties (including the bias) were not much affected by the low
224 potential step. The same behavior was obtained for aerobically-purified and anaerobically-purified Hnd
225 (Figure 3 and S2).

226 4.2.3 Determination of the Km for H₂

227 We measured the Michaelis constants (Km) by examining how the steady-state H₂ oxidation current
228 depends on H₂ concentration as described in Fourmond et al. (2013). The value indicated in Table 3 for
229 Hnd hydrogenase was determined from three independent experiments. The same value was obtained for
230 Hnd purified either under aerobic or anaerobic conditions.

231 4.2.4 CO and O₂ inactivation

232 As the other FeFe hydrogenases, Hnd is inactivated in the presence of gas inhibitors such as CO and O₂.
233 We determined the kinetic constants of these inhibitions and compared their values with those determined
234 for other FeFe hydrogenases previously characterized electrochemically.

235 **4.2.4.1 CO inactivation**

236 CO inactivation was studied using the method described previously (Baffert et al., 2011): small aliquots
237 of CO solution (1% CO and 99% Argon) were added while the current was measured at a constant electrode
238 potential (Figure 4). We determined the kinetic rate constants of CO binding k_i^{CO} and CO release k_a^{CO}
239 by fitting the model in equation 1. The inactivation rate constants (k_i^{CO}) were corrected for hydrogen
240 protection (Liebgott et al., 2010). The values are summarized in Table 3, leading to an inhibition constant
241 K_i^{CO} of 50 nM at 30 °C.

242 **4.2.4.2 O₂ inactivation**

243 Figure 5 shows a chronoamperogram during which an aliquot of O₂-saturated solution is injected at
244 $t=150$ s, then O₂ is flushed away and its concentration in the electrochemical cell decreases exponentially
245 over time. Before the injection, the decrease in current is due to the oxidative anaerobic inactivation
246 described in section 3.2.1.1. When O₂ is added, the current drops. A small part of this drop is due to
247 direct reduction of O₂ at the electrode, resulting in a negative current. This contribution was taken into
248 account in the modelling. When the dioxygen is flushed out from the solution, a small reactivation could be
249 detected. We used the following model (equation 2) to obtain the modeled curve in Figure 5. Because
250 the initial oxidative anaerobic inactivation is biphasic, the model includes two inactive species of the
251 enzyme (named inactive1 and inactive2) formed during this inactivation. Then a "dead-end" species is
252 formed by O₂ inhibition with a rate that depends on dioxygen concentration. We also tested the model with
253 partly reversible O₂ inactivation, such as described in ref (Orain et al., 2015) but it cannot be fitted to the
254 experimental data. A kinetic constant $k_{\text{in}}^{\text{O}_2}$ of 2.4 mM O₂⁻¹.s⁻¹ was obtained with the modelization (Table
255 3).

256 Similar values for CO and O₂ inhibition kinetic constants were obtained for Hnd purified either under
257 aerobic or anaerobic conditions.

5 DISCUSSION

258 The conditions of purification have little influence on the enzymatic properties of the Hnd hydrogenase
259 (K_m for methyl-viologen, K_m for H₂, optimal pH and temperature). The purification conditions slightly
260 influence the activity of the enzyme. The difference in activity between anaerobically- and aerobically-
261 purified Hnd is greater with physiological partners using the electron bifurcation mechanism than with
262 artificial redox partner (MV) (Table 1). These results suggest that aerobic purification could partly damage
263 the enzyme, disrupting the complex. This would explain the decrease in H₂-oxidation activity and the
264 higher impact on the electron bifurcation reaction.

265 The catalytic and inactivation rates, *i.e.* the shape of the cyclic voltammogram in Figure 1, depend
266 on the conditions of the purification of Hnd (aerobic *vs* anaerobic). One additional inactivation process
267 occurs at lower potential for the anaerobically purified Hnd, and this inactivation disappears after one
268 day of storage (see insert of Figure 1). This indicates the presence of two forms of the enzyme that can
269 interconvert. The presence of the two forms and their interconversion hinder a full characterization of the

270 oxidative inactivation. However, the process appears to be biphasic (see Figure 5 before the addition of
271 dioxygen) with the formation of two different inactive species (named inactive1 and inactive2 in equation
272 2). The molecular difference between the two forms is still unknown, and could be due to a small change
273 in the environment of the active site, as well as a conformational change. Further characterization of the
274 interconversion and the oxidative inactivation is in progress in our laboratory.

275 Hnd is inactivated under very mild oxidative conditions compared to standard prototypic FeFe
276 hydrogenases (Del Barrio et al., 2018a), especially when Hnd is purified under anaerobic conditions.
277 This low potential oxidative inactivation was observed for two other hydrogenases: CpIII from *Clostridium*
278 *pasteurianum* (Artz et al., 2019) and CbA5H from *Clostridium beijerinckii* SM10 (Corrigan et al. (2020)
279 and T. Happe et al., unpublished). This property was attributed in CpIII hydrogenase to the lack of polar
280 residues in the vicinity of the H-cluster inducing a low dielectric permittivity (ϵ). However, by looking at
281 the amino acids in the vicinity of the H-cluster (See supplementary data S4), this conclusion is not valid for
282 CbA5H hydrogenase and even less for Hnd hydrogenase. Furthermore, considering Hnd, the kinetics of the
283 oxidative inactivation and thus the potential at which it occurs depends on the conditions of purification
284 while the amino acid composition is unchanged. This suggests that small changes around the H-cluster
285 tune the rates of the oxidative inactivation process.

286 The bias depends on the rate of catalysis in either direction considered, but also on the rate of the oxidative
287 inactivation. The two depend on temperature, thus the bias could be influenced by the temperature. As
288 shown in Table 2, the catalytic bias can change within only 10 °C, from being toward H₂-production (at 30
289 °C) to being toward H₂-oxidation (at 20 °C). Such a change has not been reported before with any enzyme.

290 In addition to oxidative inactivation, Hnd also inactivates under reducing condition (Figure 3). The shape
291 of the cyclic voltammogram after low potential inactivation is similar (only small changes are visible) to
292 the initial, unlike the case of HydA1 from *C. reinhardtii* (Hajj et al., 2014). In the case of HydA1 from *C.*
293 *reinhardtii*, the change in the shape of the cyclic voltammogram was attributed to the formation of a form
294 of the enzyme with catalytic activity different from that of the just purified enzyme. In the case of Hnd, the
295 species formed under very reductive conditions is fully inactive or the reactivation is so fast that only the
296 active enzyme is present at the beginning of the scan. Further investigations are needed to fully understand
297 this process and to understand the differences with the other FeFe hydrogenases.

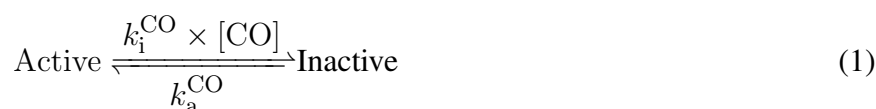
298 The Table 3 compares the kinetic constants and inhibition constant by CO and O₂ of various FeFe
299 hydrogenases, including Hnd. With a value of 0.55 bar, the K_m for H₂ is similar to that observed for the
300 other FeFe hydrogenases. It should be noted that our set-up does not allow for a pressure of H₂ greater
301 than one atm., which implies that the large value of K_m is only measured with low accuracy. CO is a
302 competitive inhibitor of H₂ oxidation by FeFe hydrogenases and H₂ has a protective effect even if the K_m
303 for H₂ is high, so we chose to consider the true inhibition constant and not the apparent inhibition constant
304 (Liebgott et al., 2010). While the reactivation kinetic constant (k_a) does not differ much from one FeFe
305 hydrogenase to the other (around 0.02 s⁻¹), the inhibition kinetic constant (k_i) is very dependent on which
306 hydrogenase is considered (Table 3 and Caserta et al. (2018)). The value obtained for Hnd is similar to that
307 observed for the HydAB hydrogenase from another *Desulfovibrio* bacterium (*D. desulfuricans*) (Liebgott
308 et al., 2010; Goldet et al., 2009) but also to that of HDCR from *Acetobacterium woodii* (Ceccaldi et al.,
309 2017). The low inhibition constant (K_i) of these three hydrogenases is probably not due to their multimeric
310 composition but rather to the CO diffusion kinetics to the H-cluster. However, the fast diffusion of CO into
311 these three hydrogenases could be attributed to a higher flexibility needed for the complex formation as
312 proposed by Marsh and Teichmann (2014).

313 Hnd retains activity even when it is purified under aerobic conditions because it forms an O₂-protected
 314 state named H_{ox}^{inact} (Kpebe et al., 2018). Recent studies showed that in the Hox-inact state, a sulfur atom
 315 binds the H-cluster: it is either exogenous sulfur (present in the culture media) in the case of "standard"
 316 hydrogenases or the sulfur atom of a cysteine in the case of *Clostridium beijerinckii* (Rodríguez-Maciá
 317 et al. (2018); Corrigan et al. (2020) and Happe, communication at the 2019 Hydrogenase conference).
 318 We recorded CVs in absence and in presence of Na₂S (Figure S6) and we observed the same effect
 319 as that described with *D. desulfuricans* while Na₂S has no effect on CbA5H FeFe-hydrogenase from
 320 *Clostridium beijerinckii*(Corrigan et al., 2020). Once it is activated under reducing conditions, it becomes
 321 sensitive to dioxygen inhibition as shown in Figure 5, which has been already observed for HydAB from
 322 *D. desulfuricans* (Roseboom et al., 2006; Rodríguez-Maciá et al., 2018). Unlike the FeFe hydrogenase
 323 CbA5H from *Clostridium beijerinckii* (Morra et al., 2016; Corrigan et al., 2020), Hnd is not converted
 324 from the active state back to the H_{ox}^{inact} state when exposed to O₂. Many FeFe hydrogenases can not form
 325 H_{ox}^{inact} state and are inhibited by O₂, either irreversibly or partially reversibly when O₂ is flushed away. In
 326 the later case, the overall O₂-sensitivity is defined by the effective inhibition rate constante k_{eff}^{O₂} (Table
 327 3) (Caserta et al., 2018). Hnd data are better fitted to the model in equation 2 with the inhibition by O₂
 328 being irreversible, and only the kinetic constant k_{in}^{O₂} can be determined. It could be directly compared to
 329 k_{eff}^{O₂}. The sensitivity to O₂ of Hnd is much higher than that of HydA1 from *C. acetobutylicum* and HydA
 330 from *M. elsdenii* but similar to that of HydA1 from *C. reinhardtii* and HDCR from *Acetobacterium woodii*.
 331 *Desulfovibrio* bacteria can face transient exposure to dioxygen under physiological conditions and indeed
 332 possess oxygen reductases (Dolla et al., 2006; Schoeffler et al., 2019). Furthermore, different enzymes
 333 from *Desulfovibrio* species were shown to resist exposure to O₂ (pyruvate:ferredoxin oxidoreductase (Vita
 334 et al., 2008) and CO-dehydrogenase (Merrouch et al., 2015)). The reversible oxidative inactivation of Hnd
 335 could be another mechanism for O₂-protection, under physiological conditions.

6 CONCLUSION

336 Here were reported the full enzymatic characterization, using biochemical and electrochemical methods, of
 337 the electron-bifurcating hydrogenase Hnd from *D. fructosovorans* purified either under aerobic or anaerobic
 338 conditions. While usually the conditions of purification are not considered (or the purification is only
 339 possible under one condition), we show that the conditions of purification could influence the properties of
 340 the enzyme: the presence or the absence of air during purification leads to two different forms of the enzyme
 341 that can interconvert. These two forms show differences in the catalytic properties, mainly regarding the
 342 oxidative inactivation. The anaerobic oxidative inactivation of one of the forms of the enzyme occurs at
 343 relatively low potential compared with other characterized hydrogenases, a behavior already observed with
 344 two other FeFe hydrogenases: CbA5H from *Clostridium beijerinckii* (Corrigan et al., 2020) and CpIII from
 345 *Clostridium pasteurianum* (Artz et al., 2019). The other catalytic properties (K_m, CO and O₂ inhibition
 346 constant) do not depend on the conditions of purification and are comparable to those of prototypic FeFe
 347 hydrogenases already characterized. This indicates that the presence of additional subunits in Hnd complex
 348 has little effect on the catalytic and inactivation properties of the hydrogenase. The characterization of the
 349 isolated hydrogenase subunit HndD is in progress to verify this hypothesis.

350 6.1 Equations



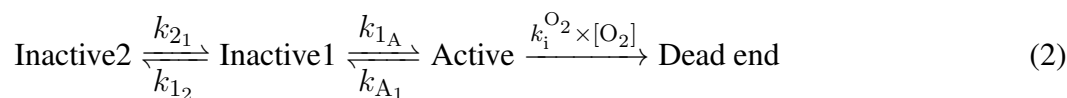
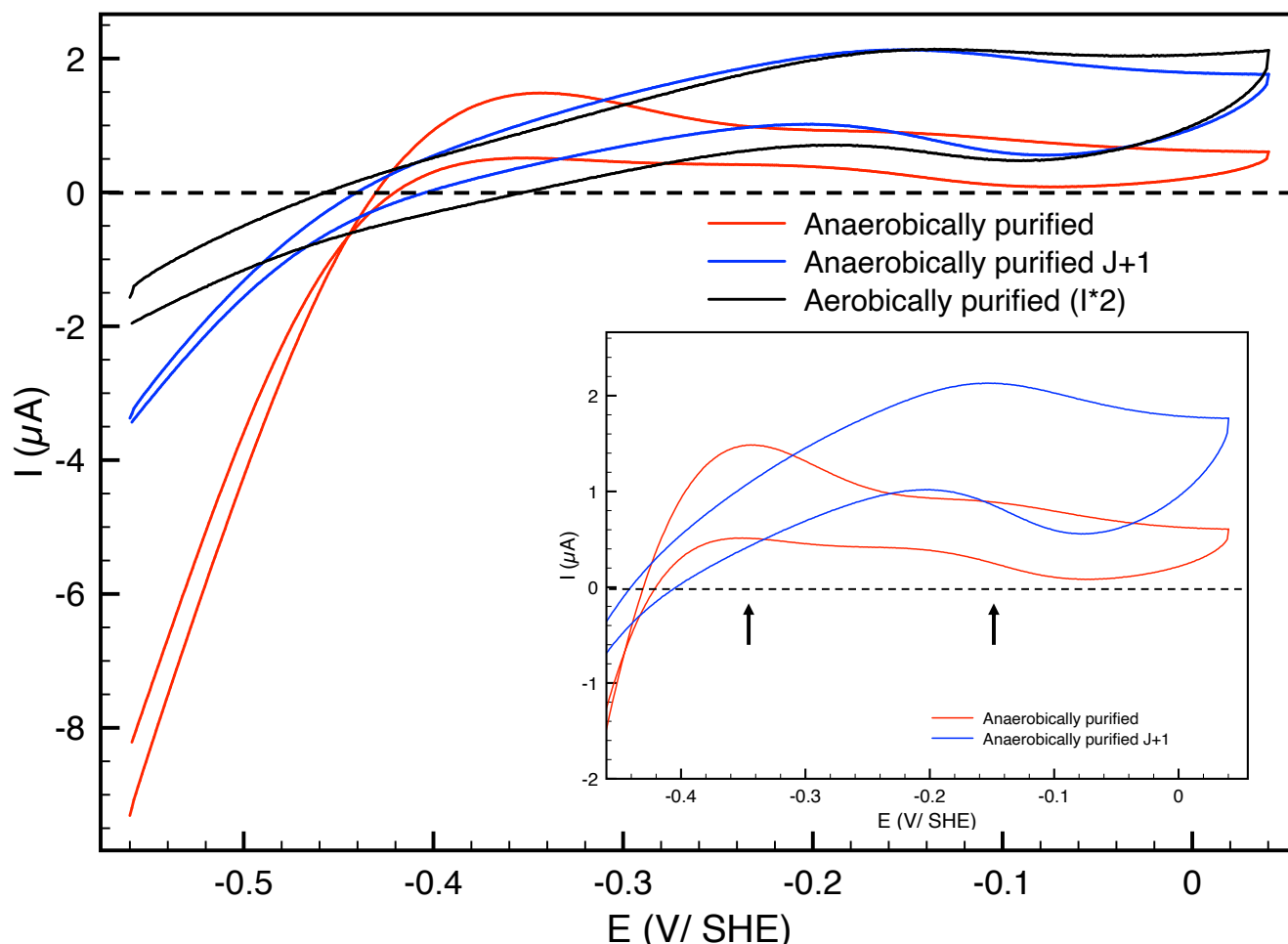
351 **6.2 Figures**

Figure 1. Cyclic voltammograms of Hnd hydrogenase adsorbed on a PGE electrode; black: aerobically-purified enzyme, red: anaerobically-purified enzyme, blue: anaerobically-purified enzyme, stored 1 day (J+1) anaerobically at 4 °C. The current for the aerobically-purified enzyme (black line) was multiplied by two for the sake of clarity. Insert: zoom in the high potential range. Scan rate: 20 mV/s, T= 30 °C, 1 bar H₂, phosphate buffer pH 7, ω= 3000 rpm

352 **6.3 Tables****7 NOMENCLATURE****CONFLICT OF INTEREST STATEMENT**

353 The authors declare that the research was conducted in the absence of any commercial or financial
354 relationships that could be construed as a potential conflict of interest.

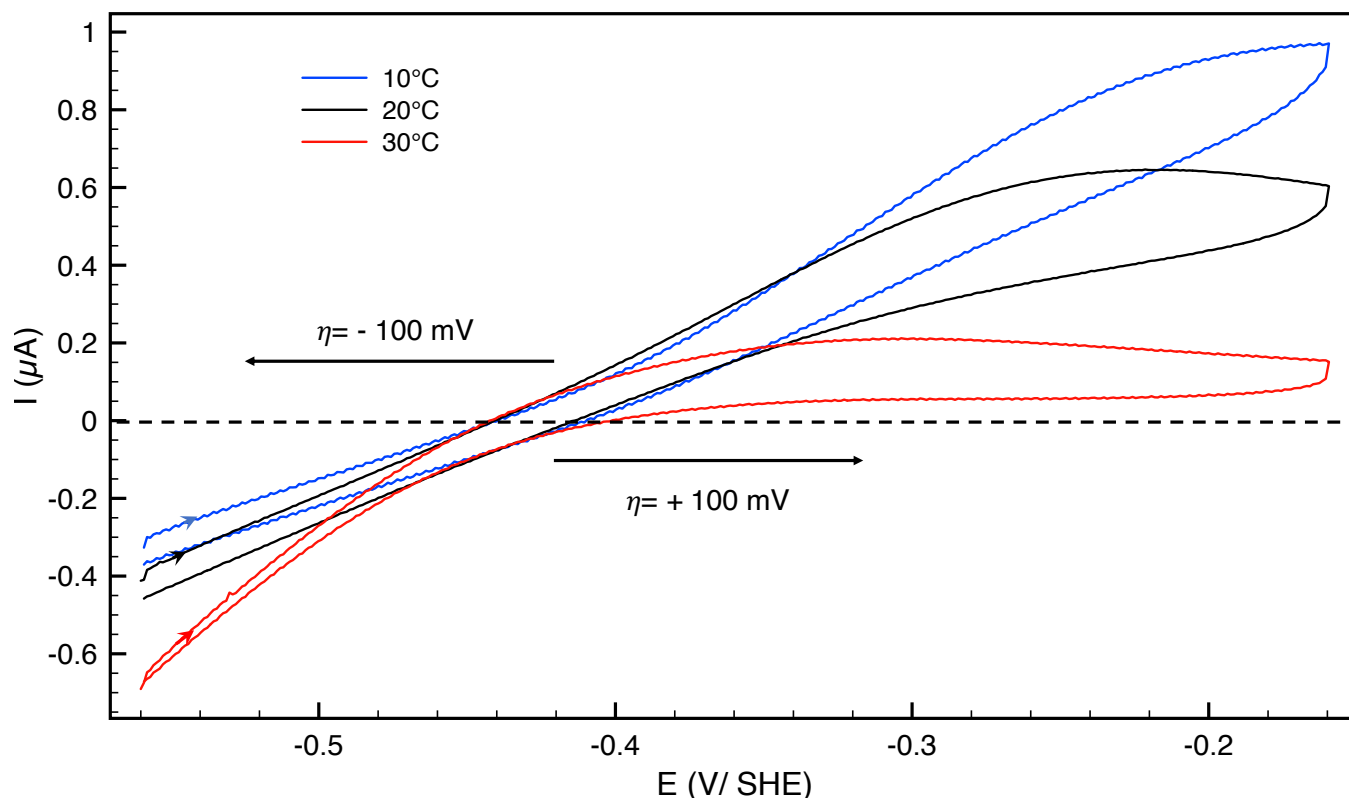


Figure 2. Cyclic voltammograms of anaerobically-purified Hnd hydrogenase adsorbed on PGE electrode as a function of temperature; blue: 10 °C, black: 20 °C, red: 30 °C. Scan rate: 20 mV/s, 1 bar H₂, phosphate buffer pH 7, ω = 3000 rpm

Purification conditions	K _m (MV) (in mM)	Optimal T (in °C)	Optimal pH	SA (MV) H ₂ oxidation (in U/mg of enzyme)	SA (MV) H ₂ production (in U/mg of enzyme)	SA (bifurcation) H ₂ oxidation (in U/mg of enzyme)
Aerobic	15 ± 2 ^a	55 ^a	8 ^a	475 ± 40	64.5 ± 13	0.21 ± 0.12
Anaerobic	13 ± 2	55	8	740 ± 45	53.5 ± 5	0.72 ± 0.3
Ratio (anaerobic/aerobic)				1.56	0.83	3.43

Table 1. Comparison of catalytic properties of Hnd purified either under aerobic or anaerobic conditions. The values are the average of three independent experiments. T= temperature, SA= specific activity. ^a Data from Kpebe et al. (2018).

AUTHOR CONTRIBUTIONS

355 AB, MB, NP, AK and CB purified the proteins and performed experiments. CF, VF and CB analyzed the
356 data. CL, MB and CB instigated the research. All the authors wrote the manuscript.

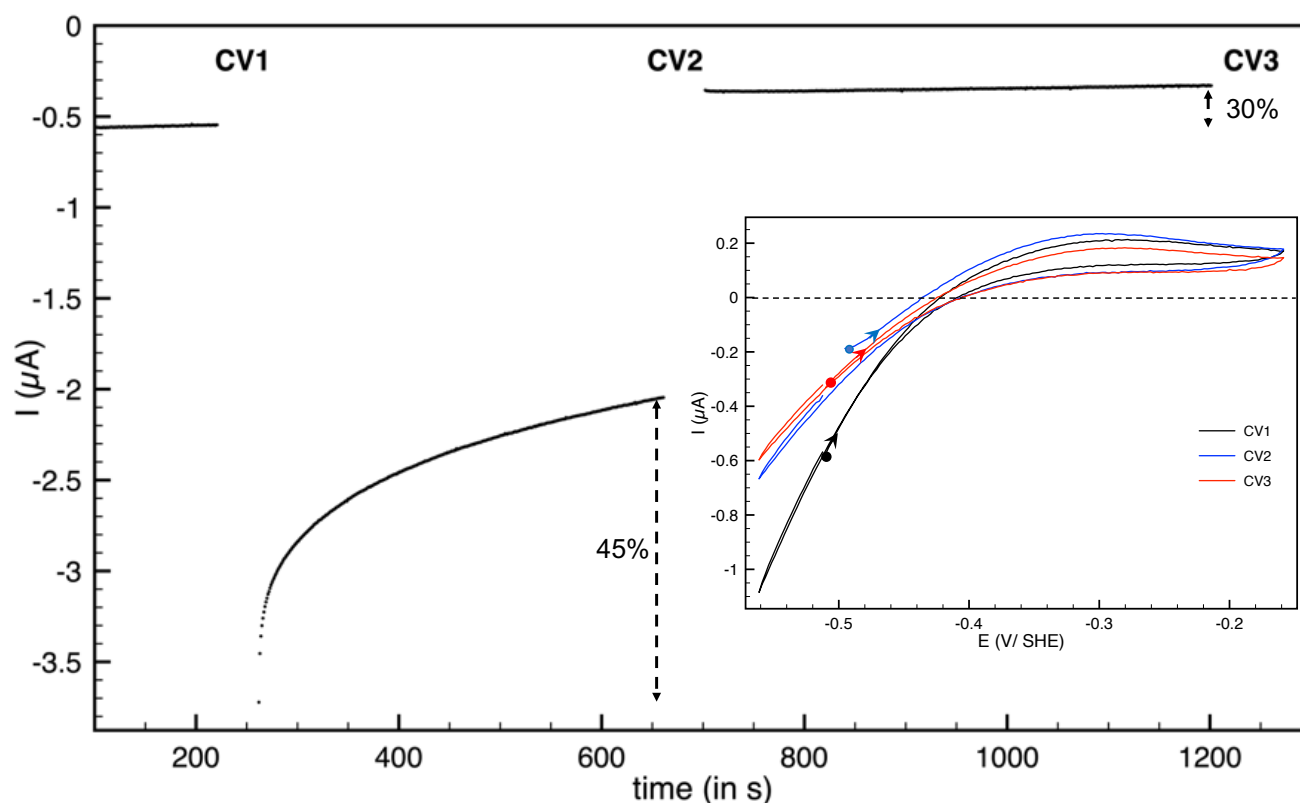


Figure 3. Reductive inactivation of aerobically-purified Hnd hydrogenase adsorbed on PGE electrode. Main: chronoamperogram, $E = -510$ mV vs SHE for $t < 250$ s and $t > 700$ s and $E = -760$ mV vs SHE for $250 < t < 700$ s. Inset: cyclic voltammograms recorded after the first potential step (CV1, black line), after the second step (CV2, blue line) and after the last step (CV3, red line). The background current was subtracted. $T = 30$ °C, 1 bar H_2 , phosphate buffer pH 7, $\omega = 3000$ rpm.

Temperature	$I(\eta=100\text{mV})$ (in μA)	$I(\eta=-100\text{mV})$ (in μA)	Bias	
			$I(\eta=100\text{mV})/I(\eta=-100\text{mV}) $ anaerobic	$I(\eta=100\text{mV})/I(\eta=-100\text{mV}) $ aerobic
30°C	0.20	-0.45	0.44	0.21
20°C	0.50	-0.30	1.67	1.25
10°C	0.55	-0.20	2.75	1.93

Table 2. Catalytic bias data extracted from Figure 2 and Figure S1, for an overpotential $\eta = \pm 100$ mV.

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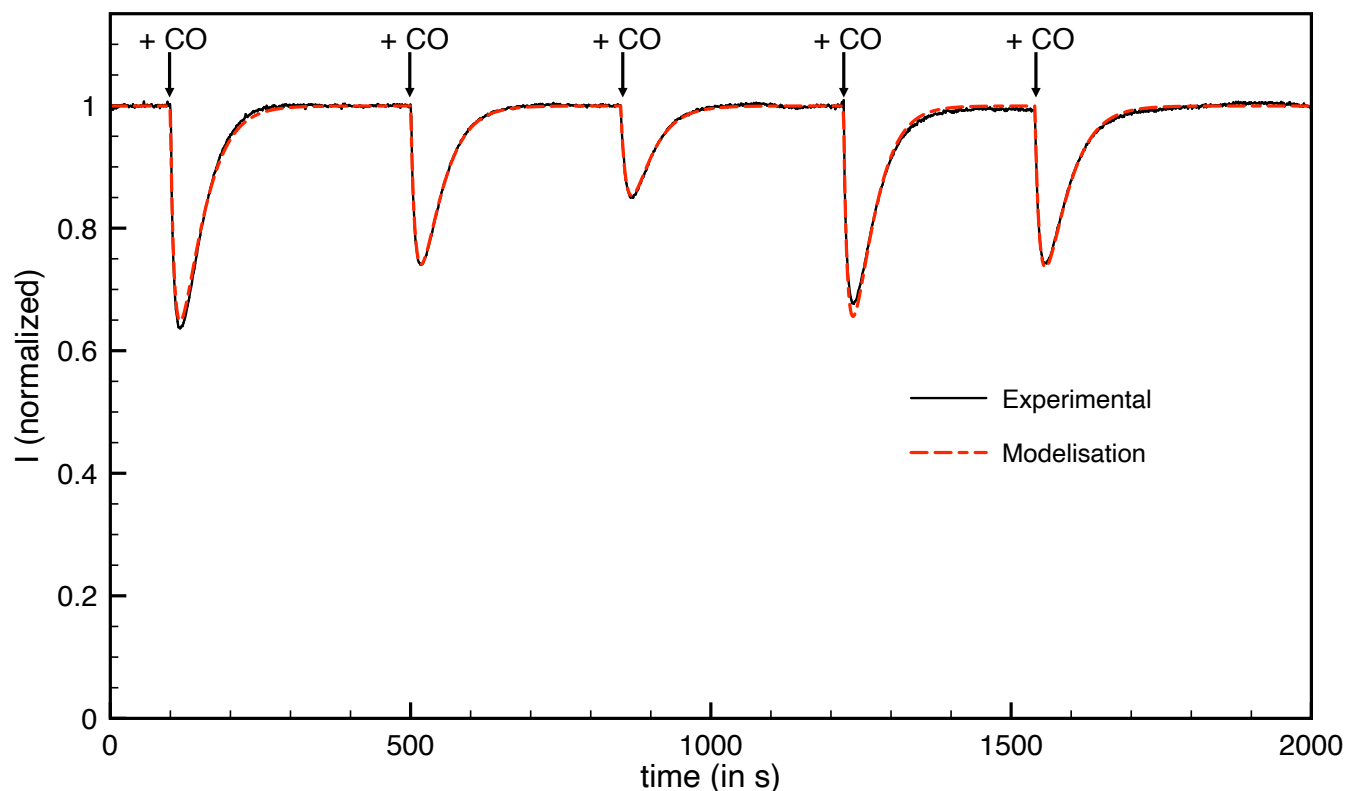


Figure 4. Inactivation by CO of aerobically-purified Hnd hydrogenase adsorbed on PGE electrode. The black line corresponds to experimental data and the dashed red line corresponds to best fit of the model in equation 1. [CO]= 288 nM, 192 nM, 96 nM, 288 nM and 192 nM injected at respectively $t= 100$ s, 500 s, 850 s, 1220 s and 1540 s; $E= -360$ mV vs SHE, $T=30$ °C, 1 bar H_2 , phosphate buffer pH 7, $\omega= 3000$ rpm.

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SUPPLEMENTAL DATA

367 S1: Cyclic voltammograms of aerobically-purified Hnd hydrogenase adsorbed on PGE electrode as
 368 a function of temperature; S2: Reductive inactivation of anaerobically and aerobically-purified Hnd
 369 hydrogenases; S3: Parameters used for the modelization of Figure 5; S4: Amino-acids in the vicinity of
 370 the H-cluster for several hydrogenases; S5: successive cyclic voltammograms of aerobically-purified Hnd
 371 hydrogenase; S6: Cyclic voltammograms of aerobically-purified Hnd hydrogenase without and with 1mM
 372 Na_2S ; S7: Specific activity and biochemical and catalytic properties of different enzyme preparation.

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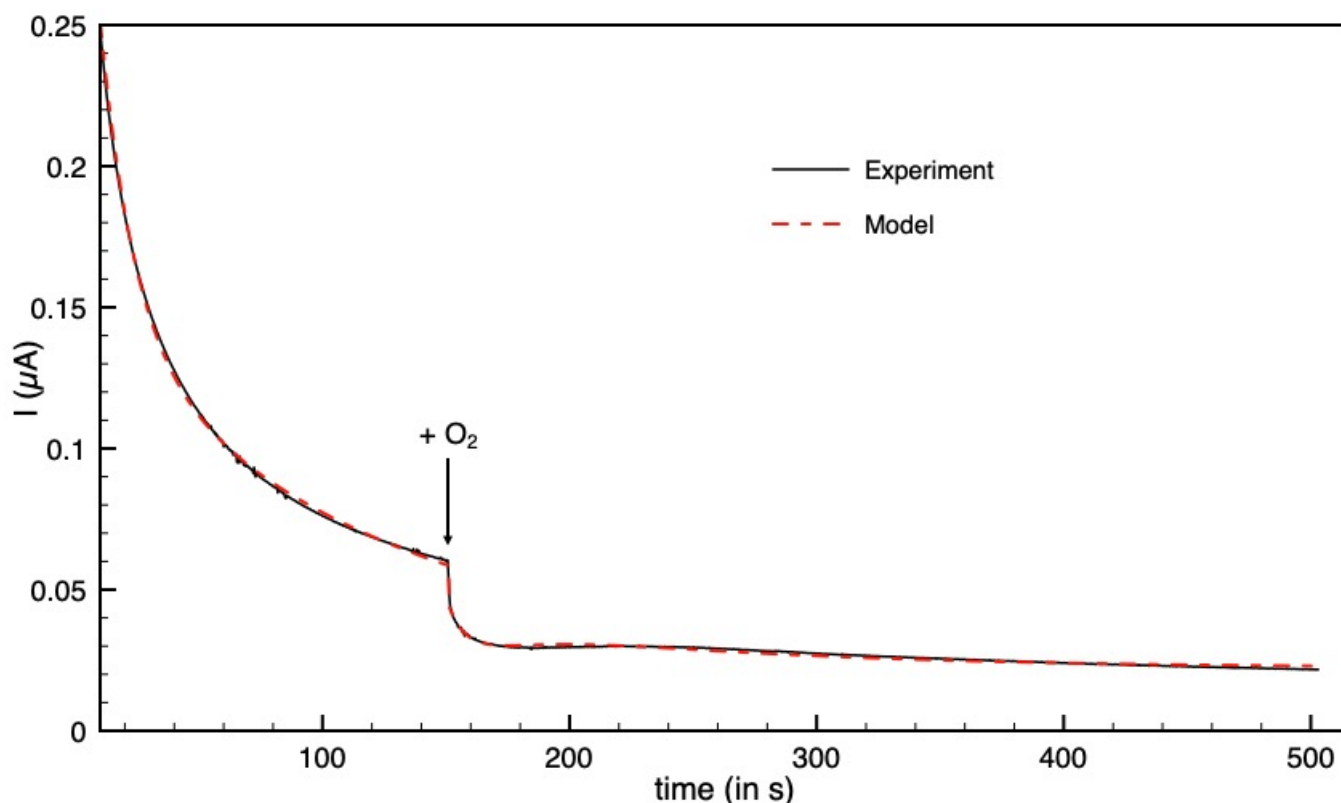


Figure 5. Inactivation by O_2 of aerobically-purified Hnd hydrogenase adsorbed on PGE electrode. The black line corresponds to experimental data and the dashed red line is the best fit of the model in equation 2. $[O_2]= 48 \mu M$ injected at $t= 150$ s. $\text{textitE}= +40$ mV vs SHE, $T=12$ °C, 1 bar H_2 , phosphate buffer pH 7, $\omega= 3000$ rpm.

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Enzyme	K _m (H ₂) (in bar)	k _i ^{CO} (in mM CO ⁻¹ .s ⁻¹)	k _a ^{CO} (in s ⁻¹)	K _i ^{CO} (in mM CO)	k _{eff} ^{O₂} (in mM O ₂ ⁻¹ .s ⁻¹)	k _{in} ^{O₂} (in mM O ₂ ⁻¹ .s ⁻¹)
<i>C.a.</i> HydA	0.8 (30°C) ^a	8 (30°C) ^b	0.03 (30°C) ^c	3.75 × 10 ⁻³	0.05 (20°C) ^b ; 0.077 (12°C) ^d	0.9 (20°C) ^b ; 1.1 (12°C) ^d
<i>C.r.</i> HydA1	0.6 (30°C) ^a	80 (30°C) ^b	0.015 (30°C) ^c	1.9 × 10 ⁻⁴	1.02 (12°C) ^d	2.5 (12°C) ^d
<i>D.d.</i> HydAB	0.27 (30°C) ^e	1000 (30°C) ^e	0.03 (30°C) ^e	3 × 10 ⁻⁵	n.d.	40 (30°C) ^e
<i>M.e.</i> HydA	0.58 (5°C)	2 (20°C) ^b	0.003 (20°C) ^b	4.5 × 10 ⁻³	0.075 (20°C) ^b	0.25 (20°C) ^b
<i>A.w.</i> HDCR	0.24 (30°C) ^f	930 (30°C) ^f	0.02 (30°C) ^f	2.1 × 10 ⁻⁵	2.5 (30°C) ^f	6.5 (30°C) ^f
<i>D.f.</i> HndABCD	0.55 ± 0.15 (30°C) ^g	1000 ± 340 (30°C) ^g	0.05 ± 0.02 (30°C) ^g	5 × 10 ⁻⁵	n. d.	2.4 ± 1.6 (12°C) ^g

Table 3. Comparison of the kinetic parameters determined by electrochemistry of different FeFe hydrogenases, including Hnd.^a (Fourmond et al., 2013); ^b (Caserta et al., 2018); ^c (Baffert et al., 2011), ^d (Orain et al., 2015), ^e (Liebgott et al., 2010); ^f (Ceccaldi et al., 2017); ^g this study, the values are the average of three independent experiments both for Hnd purified under aerobic or under anaerobic conditions. The k_i^{CO} values are corrected for the effect of hydrogen protection (Liebgott et al., 2010). K_i^{CO} = k_a^{CO}/k_i^{CO}. *C.a.* = *Clostridium acetobutylicum*, *C.r.* = *Chlamydomonas reinhardtii*, *D.d.* = *Desulfovibrio desulfuricans*, *M.e.* = *Megasphaera elsdenii*, *A.w.* = *Acetobacterium woodii*, *D.f.* = *Desulfovibrio fructosovorans*.

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