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Aurore Jacq-Bailly, Martino Benvenuti, Natalie Payne, Arlette Kpebe, Christina Felbeck, et al.. Electrochemical characterization of a complex FeFe hydrogenase, the electron-bifurcating Hnd from Desulfovibrio fructosovorans. Frontiers in Chemistry, 2021, 10.3389/fchem.2020.573305. hal-03103487

HAL Id: hal-03103487 https://hal.science/hal-03103487

Submitted on 8 Jan 2021

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

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2 ABSTRACT

Hnd, an FeFe hydrogenase from *Desulfovibrio fructosovorans*, is a tetrameric enzyme that can 3 perform flavin-based electron bifurcation. It couples the oxidation of H₂ to both the exergonic 4 reduction of NAD⁺ and the endergonic reduction of a ferredoxin. We previously showed that Hnd 5 retains activity even when purified aerobically unlike other electron-bifurcating hydrogenases. 6 In this study, we describe the purification of the enzyme under O₂-free atmosphere and its 7 biochemical and electrochemical characterization. Despite its complexity due to its multimeric 8 composition, Hnd can catalytically and directly exchange electrons with an electrode. We 9 characterized the catalytic and inhibition properties of this electron-bifurcating hydrogenase 10 using protein film electrochemistry of Hnd by purifying Hnd aerobically or anaerobically, then 11 12 comparing the electrochemical properties of the enzyme purified under the two conditions via protein film electrochemistry. Hydrogenases are usually inactivated under oxidizing conditions in 13 the absence of dioxygen and can then be reactivated, to some extent, under reducing conditions. 14 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 (Km 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

27 2.1 Electron-bifurcating enzymes

Oxidoreductase enzymes usually catalyze electron transfer between one electron donor and one 28 29 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 30 acceptors. More importantly, the reactions with the two electron donors or the two electron acceptors are 31 32 thermodynamically coupled, one of the reactions being exergonic and the other endergonic. The global 33 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 bc_1 complex in which 34 the electron-bifurcating site is a quinone (Mitchell, 1975). More recently, electron bifurcating enzymes 35 were described in which the electron-bifurcation site is a flavin (Herrmann et al., 2008). The common 36 feature of quinones and flavins is their two redox transitions, making them 2-electron centers. A variety 37 38 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+ 39 40 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 41 42 bioenergetic of many organisms, but this mechanism is still poorly understood, in part due to the low number of model enzymes characterized to date. 43

44 2.2 Electron-bifurcating hydrogenases

45 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., 46 2016; Greening et al., 2016). They are all of FeFe-type and consist of at least one subunit harboring the 47 catalytic H-cluster (the hydrogenase active site, which consists of a [4Fe4S] cluster bound via a cysteine 48 49 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 50 reduction of both NAD⁺ and a ferredoxin with a bifurcation mechanism and/or the reduction of proton 51 coupled to oxidation of NADH and a ferredoxin with a confurcation mechanism. So far, electron-bifurcating 52 hydrogenases from five anaerobic bacteria have been purified and characterized (Schuchmann and Mueller, 53 2012; Wang et al., 2013; Zheng et al., 2014), including the electron-bifurcating hydrogenase HydABC 54 from Thermotoga maritima (Schut and Adams, 2009) and HndABCD from Desulfovibrio fructosovorans 55 (Kpebe et al., 2018). They have been tested for either electron bifurcation, or electron confurcation or for 56 57 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 58 these enzymes are still controversial subjects. Further characterization of electron bifurcating hydrogenases 59 that could be models of this class of enzyme, will increase the understanding of the overall mechanism of 60 electron bifurcation. 61

62 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*

(Parkin et al., 2006; Vincent et al., 2005; Goldet et al., 2009; Rodríguez-Maciá et al., 2018), HydA from 68 69 Clostridium acetobutylicum (Baffert et al., 2008; Goldet et al., 2009; Baffert et al., 2011, 2012; Kubas et al., 2017; Orain et al., 2015), HydA1 from Chlamydomonas reinhardtii (Stripp et al., 2009; Goldet et al., 70 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 Solobacterium moorei (Land et al., 2019). All these FeFe hydrogenases are prototypical A1 monomeric or 73 dimeric enzymes (Søndergaard et al., 2016). Different aspects of the catalytic properties of these enzymes 74 are studied by electrochemical methods: affinity for the substrate (Km), effect of pH on the catalytic 75 76 properties, kinetics of inhibition by small molecules (CO, O₂, S₂⁻, formaldehyde), kinetics of oxidative and reductive inactivation, and catalytic bias. Only recently was a multimeric Hydrogen Dependent Carbon 77 Dioxide Reductase (HDCR) from Acetobacterium woodii characterized by electrochemistry (Ceccaldi 78 79 et al., 2017). This hydrogenase is classified A4. It consists of four subunits: the hydrogenase subunit hosting the H-cluster, the formate dehydrogenase subunit hosting the Mo-bisPGD cofactor and two subunits 80 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 of specific amino acids) give insight into the catalytic mechanism of FeFe hydrogenases and the molecular 83 determinants of the inactivations. The second and last multimeric hydrogenase characterized by direct 84 electrochemistry belongs to the electron-bifurcating enzyme family (classified A3), the hydrogenase 85 HydABC from *Thermotoga maritima* (Chongdar et al., 2020). The enzyme is heterologously produced in 86 87 Escherichia coli and artificially matured with a synthetic diiron cofactor. The isolated hydrogenase catalytic subunit, as well as the complex HydABC, are adsorbed onto an electrode and cyclic voltammograms are 88 recorded at various pH, to show that the isolated hydrogenase subunit and the trimeric complex behave in a 89 90 similar fashion.

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

3 MATERIAL AND METHODS

97 3.1 Enzyme purification

The production and the purification of Hnd hydrogenase from *Desulfovibrio fructosovorans* under aerobic 98 conditions were previously described (Kpebe et al., 2018). The procedure was modified to maintain 99 100 anaerobic conditions: all steps were performed in a glove-box (Jacomex, $[O_2] \le 2$ ppm) except the ultra-centrifugation step, for which anaerobiosis is maintained in the tube due to an airtight plug. The 101 cell lysis was performed by sonication (10 cycles of 30 seconds) and the Strep-tagged hydrogenase was 102 purified on a StrepTactin- Superflow (IBA) column (20 mL). Purification was done according to the 103 104 manufacturer's instructions. For the comparison of the catalytic properties, the bacterial culture was split in two, and purifications of the Hnd hydrogenase were performed in parallel under both anaerobic and aerobic 105 106 conditions.

107 3.2 Catalytic activity determination

108 All assays were performed at 30 $^{\circ}$ 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 H_2 of 1 bar, in 800 μ L 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 (ε = 13,600 M⁻¹·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

- 114 Elmer), between 10 and 200 ng of purified Hnd were added to the mixture to start the 115 hydrogenase activity corresponds to the uptake of 1 μ mol 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

by the addition of 0.3 to 1.1 μ g of purified Hnd. One unit of hydrogenase activity corresponds to the

122 production of 1 μ mol of H₂/min.

123 3.2.3 Electron-bifurcating activity for H₂ production

Electron-bifurcating (NAD⁺- and Fd-dependent) H₂-oxidizing activity was assayed as described 124 125 previously (Kpebe et al., 2018): in anaerobic quartz cuvettes, under 1 bar of H₂, in 800 μ L-mixture containing 100 mM Tris-HCl pH 8.0, 5 µM of FMN, 5 µM of FAD, and 3 mM NAD⁺ in the presence of 20 126 µM of purified FdxB ferredoxin from D. fructosovorans. NAD+- and FdxB-reduction were followed 127 128 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 410 nm respectively using the QSoas software (Fourmond, 2016), an open source program available at 130 www.qsoas.org. The specific activity is given in μ mol of NADH/min/mg. The absorption coefficients used 131 were: ε (NADH) = 6320 M⁻¹·cm⁻¹, ε (FdxB_{ox}410nm) = 24,000M⁻¹·cm⁻¹ and ε (FdxB_{red}410 nm) = 12,000 132 $M^{-1} \cdot cm^{-1}$ (Kpebe et al., 2018). 133

134 3.3 Electrochemical techniques

All electrochemical experiments were carried out with the electrochemical set-up and equipment described 135 in reference (Léger et al., 2004) in a glovebox (Jacomex) filled with N₂. 1 µL of Hnd enzyme solution was 136 mixed with 1 μ L of DTT 1M and 8 μ L of phosphate buffer pH 7. The final enzyme concentration was 2 to 137 7 μ M. The enzyme was adsorbed (1 μ L of the previous mix) onto a pyrolytic graphite edge electrode (PGE, 138 surface area $\approx 3 \text{ mm}^2$) previously polished with an aqueous alumina slurry (1 μ m). The electrochemical 139 cell contained a pH 7 phosphate buffer and was continuously flushed with pure H₂ or with argon. The 140 temperature was regulated to the desired values by circulating water in the double jacket of the cell. For 141 measuring the rates of inhibition by O₂ or CO, a stock of a buffer saturated by 100% O₂ or 1% CO in 142 99% Argon was kept in a capped serum bottle and small aliquots of this solution were injected into the 143 electrochemical cell using gas-tight syringes. The concentrations of O₂ and CO were calculated using 144 the Henry's law constants: 1.25 mM (atm O_2)⁻¹ and 1 mM (atm CO)⁻¹. The change in H₂ solubility is 145 only about 12% between 10 °C and 30 °C (Wilhelm et al., 1977). This variation induces a difference in 146 the potential of H^+/H_2 Nernst couple of 2 mV. With the surface area (around 5 mm²) of the electrode, 147 the maximum current (limitation by mass transport) is 100 μ A. In the experiments of this study, the H₂ 148 oxidation current (maximum 2 μ A) is not limited by mass transport (or less than 2%) (Merrouch et al., 149 2017). The data were analyzed using the QSoas software (Fourmond, 2016). The protein film loss was 150

included in the data analysis of Figure 4 according to reference(Fourmond et al., 2009). The effect of filmloss 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 for methyl-viologen, optimal temperature and pH). If we compare the ratio of H₂ oxidation activity for the 158 two enzymes (anaerobically- and aerobically-purified Hnd) with methyl-viologen and with physiological 159 partners, the ratio is higher when considering the electron bifurcation activity. It should be noted that 160 the activities are lower than those previously reported (Kpebe et al., 2018) because experiments were 161 performed with enzyme purified from cells grown for a longer period (2 months, Covid19 lockdown period) 162 163 and at lower temperature (20 $^{\circ}$ C), and the enzyme samples were stored one week in liquid nitrogen before electron-bifurcating activity measurements. We already observed that the enzyme activity decreases quickly 164 165 after purification (by a factor of 10) and then stabilizes (Kpebe et al., 2018). However, we checked that only the specific activity changes upon storage and not the other biochemical and catalytic properties (See 166 Table S7). 167

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 under aerobic and anaerobic conditions, and compared the catalytic and inactivation kinetic properties. 179

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

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

196 4.2.1.2 Catalytic bias

The catalytic bias (or catalytic preference (Fourmond et al., 2019; Del Barrio et al., 2018b; Sensi et al., 197 2017)) is defined as the propensity of a catalyst to catalyze a reaction faster in one direction than in the 198 other. For the same positive and negative overpotential, the activity of the enzyme (i.e. the absolute value 199 200 of the current in electrochemical experiment) must be compared. The catalytic bias is mainly the function of the rate limiting step of the catalyzed reaction. When inactivation happens, it influences the bias. As 201 shown in Figure 1 anaerobically-purified Hnd is biased toward hydrogen production because it inactivates 202 203 while oxidizing H_2 . Because inactivation and catalytic rate constants vary with temperature, the bias can be modulated by changing the temperature, as illustrated in Figure 2. While anaerobically-purified Hnd is 204 biased toward proton reduction at 30 °C, the opposite is true at 10 °C (See data in Table 2 for a chosen 205 overpotential of $\eta = \pm 100$ mV, the current of the forward scan was considered). The same behavior was 206 obtained for aerobically-purified and anaerobically-purified Hnd (Figure 2, Figure S1 and Table 2). The 207 same behavior was obtained for aerobically- and anaerobically-purified Hnd (Figure 2, Figure S1 and 208 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 209 Figure 2 (-0.56 to -0.16 V); thus the second inactivation that happened around -0.15 V is not visible in 210 Figure 2. 211

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 was changed in a 3-step-sequence $(E_1-E_2-E_1)$. E_1 and E_2 values were chosen such as no activation nor 215 216 inactivation occurs at electrode-potential E_1 , 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 -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, 218 219 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 inactivation is mostly irreversible. No reactivation was detected during this third step but fast inactivation 221 222 must take place during CV2. The shape of the cyclic voltammogram after low potential inactivation is similar to the initial, thus the catalytic properties (including the bias) were not much affected by the low 223 potential step. The same behavior was obtained for aerobically-purified and anaerobically-purified Hnd 224 (Figure 3 and S2). 225

226 4.2.3 Determination of the Km for H₂

We measured the Michaelis constants (Km) by examining how the steady-state H_2 oxidation current depends on H_2 concentration as described in Fourmond et al. (2013). The value indicated in Table 3 for Hnd hydrogenase was determined from three independent experiments. The same value was obtained for Hnd purified either under aerobic or anaerobic conditions.

231 4.2.4 CO and O₂ inactivation

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

235 4.2.4.1 CO inactivation

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

242 **4.2.4.2 O**₂ inactivation

Figure 5 shows a chronoamperogram during which an aliquot of O₂-saturated solution is injected at 243 t=150 s, then O₂ is flushed away and its concentration in the electrochemical cell decreases exponentially 244 over time. Before the injection, the decrease in current is due to the oxidative anaerobic inactivation 245 246 described in section 3.2.1.1. When O_2 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 account in the modelling. When the dioxygen is flushed out from the solution, a small reactivation could be 248 detected. We used the following model (equation 2) to obtain the modelized curve in Figure 5. Because 249 the initial oxidative anaerobic inactivation is biphasic, the model includes two inactive species of the 250 enzyme (named inactive1 and inactive2) formed during this inactivation. Then a "dead-end" species is 251 formed by O_2 inhibition with a rate that depends on dioxygen concentration. We also tested the model with 252 partly reversible O_2 inactivation, such as described in ref (Orain et al., 2015) but it cannot be fitted to the experimental data. A kinetic constant $k_{in}^{O_2}$ of 2.4 mM $O_2^{-1}.s^{-1}$ was obtained with the modelization (Table 253 254 3). 255

Similar values for CO and O_2 inhibition kinetic constants were obtained for Hnd purified either under aerobic or anaerobic conditions.

5 DISCUSSION

The conditions of purification have little influence on the enzymatic properties of the Hnd hydrogenase (Km for methyl-viologen, Km for H_2 , optimal pH and temperature). The purification conditions slightly influence the activity of the enzyme. The difference in activity between anaerobically- and aerobicallypurified Hnd is greater with physiological partners using the electron bifurcation mechanism than with artificial redox partner (MV) (Table 1). These results suggest that aerobic purification could partly damage the enzyme, disrupting the complex. This would explain the decrease in H_2 -oxidation activity and the higher impact on the electron bifurcation reaction.

The catalytic and inactivation rates, *i.e.* the shape of the cyclic voltammogram in Figure 1, depend on the conditions of the purification of Hnd (aerobic *vs* anaerobic). One additional inactivation process occurs at lower potential for the anaerobically purified Hnd, and this inactivation disappears after one day of storage (see insert of Figure 1). This indicates the presence of two forms of the enzyme that can interconvert. The presence of the two forms and their interconversion hinder a full characterization of the oxidative inactivation. However, the process appears to be biphasic (see Figure 5 before the addition of
dioxygen) with the formation of two different inactive species (named inactive1 and inactive2 in equation
2). The molecular difference between the two forms is still unknown, and could be due to a small change
in the environment of the active site, as well as a conformational change. Further characterization of the
interconversion and the oxidative inactivation is in progress in our laboratory.

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

The bias depends on the rate of catalysis in either direction considered, but also on the rate of the oxidative inactivation. The two depend on temperature, thus the bias could be influenced by the temperature. As shown in Table 2, the catalytic bias can change within only 10 °C, from being toward H₂-production (at 30 °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. reinhardtii, the change in the shape of the cyclic voltammogram was attributed to the formation of a form 293 294 of the enzyme with catalytic activity different from that of the just purified enzyme. In the case of Hnd, the species formed under very reductive conditions is fully inactive or the reactivation is so fast that only the 295 active enzyme is present at the beginning of the scan. Further investigations are needed to fully understand 296 297 this process and to understand the differences with the other FeFe hydrogenases.

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

313 Hnd retains activity even when it is purified under aerobic conditions because it forms an O₂-protected state named H_{ox}^{inact} (Kpebe et al., 2018). Recent studies showed that in the Hox-inact state, a sulfur atom 314 binds the H-cluster: it is either exogenous sulfur (present in the culture media) in the case of "standard" 315 316 hydrogenases or the sulfur atom of a cysteine in the case of *Clostridium beijerinckii* (Rodríguez-Maciá et al. (2018); Corrigan et al. (2020) and Happe, communication at the 2019 Hydrogenase conference). 317 We recorded CVs in absence and in presence of Na₂S (Figure S6) and we observed the same effect 318 as that described with D. desulfuricans while Na2S has no effect on CbA5H FeFe-hydrogenase from 319 320 *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 from the active state back to the H_{ox}^{inact} state when exposed to O₂. Many FeFe hydrogenases can not form 324 Hox inact state and are inhibited by O2, either irreversibly or partially reversibly when O2 is flushed away. In 325 the later case, the overall O_2 -sensitivity is defined by the effective inhibition rate constante $k_{eff}^{O_2}$ (Table 326 3) (Caserta et al., 2018). Hnd data are better fitted to the model in equation 2 with the inhibition by O₂ 327 being irreversible, and only the kinetic constant $k_{in}^{O_2}$ can be determined. It could be directly compared to 328 k_{eff}O₂. The sensitivity to O₂ of Hnd is much higher than that of HydA1 from *C. acetobutylicum* and HydA 329 from M. elsdenii but similar to that of HydA1 from C. reinhardtii and HDCR from Acetobacterium woodii. 330 Desulfovibrio bacteria can face transient exposure to dioxygen under physiological conditions and indeed 331 332 possess oxygen reductases (Dolla et al., 2006; Schoeffler et al., 2019). Furthermore, different enzymes 333 from *Desulfovibrio* species were shown to resist exposure to O_2 (pyruvate:ferredoxin oxidoreductase (Vita et al., 2008) and CO-dehydrogenase (Merrouch et al., 2015)). The reversible oxidative inactivation of Hnd 334 could be another mechanism for O₂-protection, under physiological conditions. 335

6 CONCLUSION

Here were reported the full enzymatic characterization, using biochemical and electrochemical methods, of 336 the electron-bifurcating hydrogenase Hnd from D. fructosovorans purified either under aerobic or anaerobic 337 conditions. While usually the conditions of purification are not considered (or the purification is only 338 339 possible under one condition), we show that the conditions of purification could influence the properties of the enzyme: the presence or the absence of air during purification leads to two different forms of the enzyme 340 341 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 relatively low potential compared with other characterized hydrogenases, a behavior already observed with 343 344 two other FeFe hydrogenases: CbA5H from Clostridium beijerinckii (Corrigan et al., 2020) and CpIII from Clostridium pasteurianum (Artz et al., 2019). The other catalytic properties (Km, CO and O₂ inhibition 345 constant) do not depend on the conditions of purification and are comparable to those of prototypic FeFe 346 347 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

Active
$$\underset{k_{a}^{CO}}{\overset{k_{i}^{CO} \times [CO]}{\overset{}}}$$
Inactive (1)

Inactive 2
$$\xrightarrow{k_{2_1}}$$
 Inactive 1 $\xrightarrow{k_{1_A}}$ Active $\xrightarrow{k_i^{O_2} \times [O_2]}$ Dead end (2)

2 0 Anaerobically purified Anaerobically purified J+1 Aerobically purified (I*2) -2 2 (*MA*) -4 (Au) C -6 Anaerobically purified -8 Anaerobically purified J+1 -2 -0.4 -0.3 -0.2 -0.1 0 E (V/ SHE) -0.5 -0.4 -0.3 -0.2 -0.1 0 E (V/ SHE)

351 6.2 Figures

Figure 1. Cyclic voltammograms of Hnd hydrogenase adsorbed on a PGE electrode; black: aerobicallypurified 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	Km (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

AB, MB, NP, AK and CB purified the proteins and performed experiments. CF, VF and CB analyzed the 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. Insert: 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 H₂, phosphate buffer pH 7, ω = 3000 rpm.

Temperature	$I(\eta=100\text{mV})$ (in μ A)	$I(\eta=-100\text{mV})$ (in μ A)	Bia I(η=100mV)/ I anaerobic	as $(\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.

FUNDING

NP is funded by Doc2amu program which has received funding from the European Union's Horizon 2020 357 research and innovation programme under the Marie Sklodowska-Curie grant agreement No713750. Also, 358 359 Doc2amu has been carried out with the financial support of the Regional Council of Provence-Alpes-Cote d'Azur and with the financial support of the A*MIDEX (nº ANR-11-IDEX-0001-02), funded by the 360 Investissements d'Avenir project funded by the French Government, managed by the French National 361 Research Agency (ANR). This work was funded by the French National Research Agency (ANR-12-362 BS08-0014, ANR-14-CE05-0010) and by the A*MIDEX funded by the Investissements d'Avenir project 363 (ANR-11-IDEX-0001-02). 364



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 H₂, phosphate buffer pH 7, ω = 3000 rpm.

ACKNOWLEDGMENTS

The authors thank I. Meynial-Salles and P. Soucaille for kindly providing the plasmid pthl-Fd-LL-C-Tag.The authors are part of the French bioinorganic chemistry network (www. frenchbic.cnrs.fr).

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 Na₂S; S7: Specific activity and biochemical and catalytic properties of different enzyme preparation.

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Figure 5. Inactivation by O₂ 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. $[O_2]=48 \ \mu\text{M}$ injected at t= 150 s. textitE= +40 mV vs SHE, T=12 °C, 1 bar H₂, phosphate buffer pH 7, ω = 3000 rpm.

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Enzyme	Km (H ₂) (in bar)	k _i ^{CO} (in mM CO ⁻¹ .s ⁻¹)	ka ^{CO} (in s ⁻¹)	K _i ^{CO} (in mM CO)	$k_{eff}^{O_2}$ (in mM $O_2^{-1}.s^{-1}$)	$k_{in}^{O_2}$ (in mM $O_2^{-1}.s^{-1}$)
C.a. HydA	0.8 (30°C) ^a	8 (30°C) ^b	0.03 (30°C) ^c	3.75×10 ⁻³	$0.05 (20^{\circ}C)^{b};$ $0.077 (12^{\circ}C)^{d}$	0.9 (20°C) ^b ; 1.1 (12°C) ^d
C.r. 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
A.w. HDCR	0.24 (30°C) ^f	930 (30°C)	0.02 (30°C) f	2.1×10 ⁻⁵	2.5 (30°C) ^f	6.5 (30°C) ^f
<i>D.f.</i> HndABCD	$\begin{array}{c} 0.55 \pm 0.15 \\ (30^{\circ}\text{C})^{\text{g}} \end{array}$	$1000 \pm 340 \ (30^{\circ}C)^{g}$	$0.05{\pm}~0.02\\(30^{\circ}{\rm C})^{\rm g}$	5×10 ⁻⁵	n. d.	$2.4 \pm 1.6 \ (12^{\circ}\text{C})^{\text{g}}$

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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