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# An allosteric redox switch involved in oxygen protection in a CO<sub>2</sub> reductase

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## Abstract

Metal-dependent formate dehydrogenases reduce CO<sub>2</sub> with high efficiency and selectivity, but are usually very oxygen sensitive. An exception is *Desulfovibrio vulgaris* W/Sec-FdhAB, which can be handled aerobically, but the basis for this oxygen tolerance was unknown. Here we show that FdhAB activity is controlled by a redox switch based on an allosteric disulfide bond. When this bond is closed, the enzyme is in an oxygen-tolerant resting state presenting almost no catalytic activity and very low formate affinity. Opening this bond triggers large conformational changes that propagate to the active site, resulting in high activity and high formate affinity, but also higher oxygen sensitivity. We present the structure of activated FdhAB and show that activity loss is associated with partial loss of the metal sulfido ligand. The redox switch mechanism is reversible *in vivo* and prevents enzyme reduction by physiological formate levels, conferring a fitness advantage during O<sub>2</sub> exposure.

## Introduction

The efficient catalytic conversion of CO<sub>2</sub> into chemicals and fuels presents a major challenge due to the intrinsic kinetic and thermodynamic stability of this molecule. Reduction of CO<sub>2</sub> is very energy demanding and requires coupled proton and electron transfer occurring at low redox potentials, which leads to strong competition from the H<sup>+</sup> reduction reaction<sup>1–3</sup>. In addition to the high energy requirements, chemical catalysis of CO<sub>2</sub> reduction has low catalytic efficiency and low product selectivity. In contrast, Nature has evolutionary tailored some enzymes to efficiently and selectively

36 convert CO<sub>2</sub> with high turnovers and without the need for very low potentials. This involves the use of hydrophobic  
37 active sites that promote CO<sub>2</sub> binding and activation, the steric control of independent proton and electron transfer  
38 and the stabilization of intermediates, towards formation of single products<sup>4</sup>.

39 One of the simplest and most interesting CO<sub>2</sub> reactions is its reduction to formate. Formate has a high energy  
40 content and can be used directly in fuel cells, as a hydrogen storage material or as a precursor for chemical synthesis<sup>5-</sup>  
41 <sup>7</sup>. Reduction of CO<sub>2</sub> to formate involves the formal transfer of a hydride (two electrons and one proton), and in nature  
42 is efficiently performed by metal-dependent formate dehydrogenases (FDHs), which achieve CO<sub>2</sub> reduction at the  
43 thermodynamic potential through the spatially separate transfer of two electrons and one proton to selectively  
44 produce formate, while fully preventing the hydrogen evolution reaction<sup>8,9</sup>. Biologically, most FDHs are tailored for  
45 formate oxidation, but metal-dependent FDHs performing CO<sub>2</sub> reduction are involved in either the reductive acetyl-  
46 CoA pathway, the most energy-efficient pathway of CO<sub>2</sub> fixation<sup>10</sup>, or in syntrophic production of formate for  
47 interspecies electron transfer<sup>11,12</sup>. Both metabolisms are present in anaerobic prokaryotes, such as methanogens,  
48 acetogens, syntrophic bacteria and sulfate reducers where we can also find the most active FDHs in CO<sub>2</sub> reduction<sup>13-</sup>  
49 <sup>15</sup>. These enzymes contain a Mo or W atom at the active site coordinated by the dithiolene of two metallopterin  
50 guanosine dinucleotide (MGD) groups, one labile sulfur ligand, and a protein-ligand (cysteine or selenocysteine  
51 (Sec))<sup>16</sup>. W-dependent FDHs are particularly active in CO<sub>2</sub> reduction due to the lower redox potential of this  
52 metal<sup>8,14,17,18</sup>, but most are typically very oxygen-sensitive. Among these, the W and Sec FdhAB enzyme from  
53 *Desulfovibrio vulgaris* Hildenborough (*D. vulgaris* H) is remarkable because it has high CO<sub>2</sub> reduction activity but can  
54 be purified and handled aerobically<sup>14</sup>, which is a major advantage for potential applications. This enzyme has a simple  
55 composition with only two subunits, the large catalytic subunit containing the deeply buried Sec/bis-MGD/W cofactor  
56 and one [4Fe-4S] center, and the small subunit containing three additional [4Fe-4S] centers responsible for electron  
57 transfer to and from the active site. *D. vulgaris* H FdhAB (FDH1) is the main FDH expressed by this organism in the  
58 presence of W and is the enzyme involved in CO<sub>2</sub> reduction during syntrophic metabolism<sup>19,20</sup>. It has high affinity and  
59 reduction activity for CO<sub>2</sub> and a remarkable robustness, making it an excellent model system that has been extensively  
60 explored in different approaches for sustainable CO<sub>2</sub> reduction<sup>21-25</sup>. However, the molecular determinants conferring  
61 oxygen tolerance to FdhAB were so far unknown.

62 Here, we report that an allosteric disulfide bond present at the surface of FdhAB is critical to its robustness by  
63 acting as a redox switch that converts the enzyme from a resting, O<sub>2</sub>-tolerant, but almost inactive state when the  
64 disulfide is closed, to the fully active, but more O<sub>2</sub>-sensitive one when the disulfide is open. Reduction of this disulfide  
65 bond leads to a series of structural changes that propagate towards the catalytic site, revealing a different active  
66 conformation and the involvement of previously unidentified residues important for catalysis. In addition, the resting  
67 (inactive) state has lower affinity for formate with a K<sub>M</sub> more than two orders of magnitude higher than the active  
68 state. O<sub>2</sub> resistance requires the keeping of the active site in the oxidised state, and the role of forming the disulfide  
69 bridge is to ensure that *in vivo* the enzyme is not reduced by physiological formate levels while the cells are transiently  
70 exposed to oxygen.

## 72 Results

73 **FdhAB activity requires reductive activation.** After aerobic isolation, *D. vulgaris* H FdhAB displays no CO<sub>2</sub> reduction  
74 and very low formate oxidation activity (Fig. 1a). Reductive activation with a thiol reducing agent (DTT or TCEP) is  
75 required for full activity (Table 1)<sup>14</sup>. A requirement for reductive activation has also been reported for other metal-  
76 dependent FDHs, even after anaerobic purification<sup>18,26,27</sup>. Remarkably, the strong reductant sodium dithionite is not  
77 capable of activating FdhAB (Fig. 1a), indicating that reduction of the metal or a low redox potential is not sufficient  
78 for FdhAB activation.

79 When anaerobically purified, FdhAB does not require reductive activation by DTT (Fig. 1c and d), being in a form that  
80 we will refer to as the active state. In contrast, when FdhAB is aerobically purified less than 40% of the activity is  
81 recovered by activation, and the process is poorly reproducible as the protein easily loses activity. We tested whether  
82 oxidation of the crude extract before purification had an effect on the activity and stability of the enzyme. We found  
83 that when the crude soluble extract was oxidized under air before purification, a much more stable protein was  
84 obtained, which was isolated in an (almost) inactive state, which we called the resting state, and which could be fully  
85 activated by DTT in a reproducible way (Fig. 1c and d). Notably, in this resting state the enzyme displays no CO<sub>2</sub>  
86 reduction activity and the K<sub>M</sub> for formate is very high, 2.5 ± 0.17 mM, two orders of magnitude higher than that of the  
87 active enzyme (16.9 ± 2.8 μM) (Table 1). **The FdhAB C845-C872 bond is a redox switch.** The previous results suggest  
88 that reduction of a disulfide bond may be involved in the activation of FdhAB. Inspection of the *D. vulgaris* H FdhAB  
89 structure shows the presence of a single disulfide bond on the enzyme surface<sup>14</sup> (Fig. 1b), which is also present in the  
90 homologous *Desulfovibrio gigas* enzyme<sup>28</sup>. In *D. vulgaris* H FdhAB this disulfide bond, between C845 and C872,  
91 connects two loops and is present in the oxidized as well as in the formate- and dithionite-reduced crystal structures  
92 previously reported<sup>14,29,30</sup>. No other cysteines are present at the surface, which could form intermolecular disulfide  
93 bonds. The C845-C872 disulfide bond is 25 Å away from the W active site, in the opposite direction to the electron  
94 transfer pathway, and it is not obvious how it influences enzyme activity. To assess the role of the C845-C872 disulfide  
95 bond in reductive activation and its influence on enzyme stability and resistance to O<sub>2</sub>, two FdhAB variants were  
96 produced: C872A and C845A. These variants present a high activity that is independent of DTT activation (Fig. 1c and  
97 1d), suggesting that without the disulfide bond the enzyme is present in an active conformation. Notably, the C872A  
98 variant displays even higher activity than the WT FdhAB (Table 1, Fig. 1c and 1d), whereas the C845A mutation has an  
99 overall negative effect on the activity. Aerobic purification of the variants is possible but leads to a decrease in activity,  
100 both for formate oxidation and CO<sub>2</sub> reduction, with or without DTT, an effect that is more evident for the C845A  
101 variant. This indicates that O<sub>2</sub> sensitivity is not determined only by the disulfide bond. The two variants have similar  
102 melting temperatures to the wild type (WT) FdhAB (79 ± 0.1 °C for C845A, 81 ± 0.1 °C for C872A versus 80 ± 0.2 °C for  
103 the WT)<sup>14</sup>, showing that cleavage of the disulfide bond does not affect the thermostability of the enzyme. In contrast  
104 to C872, residue C845 is part of the common core structure of FDH catalytic subunits (Supplementary data 1 and  
105 Supplementary Fig. 1), suggesting a possible structural role that may explain the lower activity of variant C845A.  
106 Therefore, further experiments were performed only with the C872A variant. This variant presents similar or higher

107 affinity and activity for formate or CO<sub>2</sub> conversion as the DTT-activated WT enzyme (Table 1, Extended Data Fig. 1),  
108 and so is a good proxy for the activated form of the enzyme.

109  
110 **Activated FdhAB presents a strong W<sup>V</sup> population.** The conformation of the metal active site in FDHs can be probed  
111 with great sensitivity using EPR spectroscopy that measures the W<sup>V</sup> intermediate state<sup>13,16,17,31</sup>. The EPR properties of  
112 WT FdhAB were recently characterized after reduction with dithionite or formate<sup>29</sup>. Depending on the reductant, the  
113 major species were named W<sup>V</sup><sub>D</sub> for dithionite-reduced enzyme (g = 1.993, 1.909, 1.852) (Fig. 2a) and W<sup>V</sup><sub>F</sub> for formate-  
114 reduced enzyme (g = 1.995, 1.881, 1.852) (Fig. 2b). These species differ mainly in the g<sub>2</sub>-value while g<sub>1</sub> and g<sub>3</sub> are nearly  
115 identical. This difference was interpreted as arising from only a few degrees variation of the SSSS dihedral angles of  
116 the W pterin ligands in the two forms. However, a striking observation was the much stronger intensity of the W<sup>V</sup><sub>F</sub>  
117 signal (~0.2 spins/molecule) obtained after DTT-activation and formate reduction, relative to the intensity of the  
118 unactivated dithionite-reduced form, which was not treated with DTT (~0.02 spins/molecule).

119 EPR analysis of the dithionite or formate reduced C872A variant reveals the presence of W<sup>V</sup><sub>D</sub> and W<sup>V</sup><sub>F</sub> signals,  
120 respectively, both with similar high intensity (Fig. 2c and 2d), in contrast to WT FdhAB. Reduction with formate results  
121 in a W<sup>V</sup> signal corresponding to 0.18 ± 0.02 spin/molecule (90% W<sup>V</sup><sub>F</sub>, 10% W<sup>V</sup><sub>D</sub>), and with dithionite in a more mixed  
122 signal corresponding to 0.30 ± 0.03 spin/molecule, with about 85% W<sup>V</sup><sub>D</sub> and 15% W<sup>V</sup><sub>F</sub> (Extended Data Fig. 2). This  
123 suggests that the absence of the C845-C872 bond leads to a conformational change that results in a high-intensity W<sup>V</sup>  
124 EPR signal regardless of reductant used. This is in contrast to the formate-reduced<sup>14</sup> or dithionite-reduced<sup>29</sup> forms of  
125 the resting WT enzyme with a closed disulfide bond, which presents no or very weak EPR signals. Interestingly, the  
126 two different W<sup>V</sup> signals can be present in the same sample, showing some heterogeneity in the conformation of the  
127 W ligands. The long-range conformational changes at the active site, induced by FdhAB activation (see below), are  
128 reminiscent of the remote effects observed in Nitrate reductase A, another member of the Mo/W-enzyme family,  
129 where the motion of a distant amino-acid side chain propagates through the H-bond network to the Mo-cofactor and  
130 modify its structural surrounding, redox properties and catalytic activity<sup>32</sup>. Since the only striking difference between  
131 the WT and C872A FdhAB is the increase in signal intensity, this firmly suggests that the low intensity W<sup>V</sup> signal  
132 observed in the dithionite-reduced enzyme (not activated by DTT, disulfide bond closed) is most likely derived from a  
133 small fraction of enzyme that has the disulfide bond open, and that the resting, closed state of the enzyme is EPR silent  
134 and cannot be reduced to the W<sup>V</sup> intermediate state, as previously reported for formate-reduced FdhAB without DTT  
135 activation<sup>14</sup>.

136  
137 **The crystal structure of C872A FdhAB.** The reported structures of *D. vulgaris* H FdhAB (as-isolated aerobically, formate  
138 or dithionite reduced<sup>14,29,30</sup>) all include the C845-C872 disulfide bond. Attempts to obtain a structure with a reduced  
139 disulfide bond by incubation or soaking experiments with DTT or TCEP, were unsuccessful. However, the anaerobically  
140 purified C872A variant could be crystallized in aerobic and anaerobic conditions, yielding structures C872A<sub>ox</sub> and  
141 C872A<sub>anox</sub> (the latter complexed with the substrate analogue formamide), which diffracted at 2.3 Å and 1.42 Å,  
142 respectively (Supplementary Data 2 and Supplementary Table 1). Both aerobic and anaerobic structures display

143 identical conformations being superposed with RMSD of 0.26 Å for 971 Cα of FdhA, and of 0.36 Å for 214 Cα of FdhB.  
144 In both structures the W site is fully loaded, with B-factors that match neighbouring amino acids, and the position of  
145 the MGDs, Sec192 and conserved active site residues H193 and R441 are equivalent. However, the occupancy of the  
146 sulfido ligand in the C872A\_ox structure is refined at only 56% revealing partial loss of the labile sulfur in aerobic  
147 conditions (Extended Data Fig. 3).

148 Comparing the C872A\_anox structure with the as-isolated WT FdhAB structure (PDB ID: 6SDR)<sup>14</sup> reveals that  
149 the main conformational changes are in the catalytic subunit (RMSDs of 1.10 Å for 962 Cα of FdhA and 0.34 Å for 214  
150 Cα of FdhB), close to the mutation and in domains III and IV that harbor MGD2. The lack of the disulfide bond induces  
151 a long-range conformational rearrangement propagating from the surface down to the active site (Fig. 3), with  
152 reorientation of numerous side chains and suggesting the presence of an allosteric mechanism. In the C872A\_anox  
153 structure, C845 and A872 side chains are far apart, with Cα-845 5.37 Å away from its position when the disulfide bond  
154 is present, and Cα-872 3.37 Å away (Fig. 3a). The loss of constraints imposed by the disulfide bond allows increased  
155 freedom of the neighbouring amino acids with formation of two new salt bridges (E844...R934 and E867...K564) as well  
156 as new hydrogen bonds and hydrophobic interactions. The loop F862-QIEGE-K868 that was not visible in the WT  
157 structure, is well defined in the C872A structures, and seems to push the loop S984---P993 towards the active site with  
158 the hydrophobic I992 interacting directly with the Sec192 sidechain (Fig. 3b). The new E844...R934 salt bridge at the  
159 protein surface induces a series of conformational changes that propagate to the active site, dragging L843 and P842  
160 towards the active site. These changes affect the position of the nearby loop, with a drastic movement of amino acids  
161 H410 and W407 that adopt new positions (Fig. 3c). The side chain of Q409 also moves closer to MGD2 (creating a H-  
162 bond with the pterin N15) and cause a strong shift in the side chain of M405 (Cε- Cε distance – 5.21 Å), which rotates  
163 towards the W active site causing a displacement of Sec192 that adopts a new rotamer (Fig. 3d, 3e). The movement  
164 of M405 impacts the W first and second coordination sphere, bringing the methionine Sδ closer to the Se, and MGD  
165 S1 (Fig. 3e and Supplementary Table 2). All these modifications induce a drastic rearrangement of the active site when  
166 compared with the WT FdhAB structures available (PDB 6SDR, 6SDV and 7Z5O), where the disulfide bond is present  
167 (Fig. 3d and e). In the second coordination sphere of W, catalytic R441 shows a slight deviation, but H193 moves away  
168 from the active site and presents a third conformation, not previously seen (Extended Data Fig. 4), closer to the R441  
169 side chain and opening the formate channel<sup>14</sup>. Also, both MGDs suffer a displacement that is more evident for MGD2  
170 (Fig. 3d). Interestingly, in the C872A\_anox structure one formamide molecule is found in the formate channel,  
171 hydrogen bonded to R441 and T450, likely mimicking a transit formate molecule (Extended Data Fig. 5).

172  
173 **M405 is a crucial residue in the FdhAB active site.** The conspicuous movement of M405 towards the active site upon  
174 enzyme activation suggests a key structural role of M405 in the FdhAB active site. This residue is fairly conserved in  
175 periplasmic FDHs (62% M, 27% L, 8% V)<sup>14</sup>, but is strictly conserved for Mo/W-dependent FDHs having the C845-C872  
176 motif (Supplementary Tables 3 and 4). To understand the role and possible involvement of M405 in catalysis, the  
177 M405A variant was created. This variant has virtually no activity when purified aerobically (<1%), showing no increase  
178 with DTT, and has 4-6% activity when purified anaerobically (Fig. 1c and d). Notably, the affinity for CO<sub>2</sub> of the M405A

179 variant is around 40 times lower than WT FdhAB (Table 1), while the affinity for formate is not strongly affected. Also,  
180 the optimal pH for CO<sub>2</sub> reduction is shifted to pH 6.0 instead of 7.1 for the WT enzyme (Supplementary Fig. 2), likely  
181 due to the strong decrease in CO<sub>2</sub> affinity, such that the CO<sub>2</sub> concentrations at pH 7.1 become non-saturating.

182 The anaerobically purified M405 variant, with an open disulfide bond, gave a W<sup>V</sup> EPR signal with g-values at  
183 about 2.003 ± 0.001, 1.885 ± 0.002 and 1.849 ± 0.002, close to those of the W<sup>V</sup><sub>F</sub> species observed in the C872A variant  
184 and in the activated WT FdhAB, with similar spin intensity (~0.2 ± 0.03 spin/molecule) but exhibiting much larger  
185 linewidths (Fig. 2c and Extended Data Fig. 2). This broadening likely results from a marked g-strain effect that indicates  
186 an increased distribution of conformations of the W-cofactor surroundings, but contribution of unresolved hyperfine  
187 interactions cannot be excluded. Both the shape of this signal and its intensity were not affected by a subsequent  
188 reduction with dithionite (Extended Data Fig. 6). In contrast, incubation of the variant with formate led to a weaker  
189 signal in line with its poor catalytic activity.

190 The anaerobically purified M405A variant did not crystallize, but the aerobically purified form gave suitable  
191 crystals revealing an overall fold similar to WT FdhAB (RMSD 0.41 Å for 955 Cα of FdhA and 0.29 Å for 214 Cα of FdhB),  
192 including the C845-C872 region where the disulfide bond is present. Nonetheless, notable changes are observed in the  
193 active site and its vicinity, including a pronounced conformational change of the neighbouring Q409 (conserved in  
194 FDHs with the double cysteine motif, Supplementary Table 4), which occupies the vacant space left by the mutation  
195 (Extended Data Fig. 7). This movement disturbs the active site geometry, which is partially disordered resulting in  
196 multiple orientations of the Sec192 sidechain, which exhibits weak electron density and could not be correctly  
197 modelled. The B-factors of the active site core are also relatively high, considering the high-resolution of the data, and  
198 it was not possible to track the anomalous signal of Se, or model the loop A985---I992. Thus, the substantial decrease  
199 in catalytic activity of the M405A variant can be attributed to a disturbed conformation of the coordination sphere of  
200 the W active site, caused by the absence of the M405 sidechain. This strongly suggests a key role of M405 in the  
201 allosteric mechanism involved in enzyme activation by correct positioning of the Sec192 residue, as well as in the  
202 structural integrity/geometry of the active site by stabilizing the W first coordination sphere through nonbonding  
203 interactions. These include hypervalent nonbonding S···X (X= O,N,S) interactions, as previously reported for other  
204 enzymes with a methionine close to the active site, where such interactions<sup>33</sup> were shown to impact reaction efficacy<sup>33</sup>.  
205 Indeed, several nonbonding S···X interactions involving M405 and residues close to the active site are present in the  
206 oxidized and reduced WT FdhAB structures (Supplementary Table 2). Interestingly, the C872A variant has the largest  
207 number of S···X interactions (Supplementary Table 2), since the conformational changes of M405 and Q409 led to new  
208 S···X interactions not present in the WT, further supporting the functional role of the conserved M405.

209  
210 **Oxygen tolerance of C872A FdhAB.** Aerobic purification of C872A FdhAB leads to reduced activity, but this variant is  
211 still quite stable in air, when in the oxidized W<sup>VI</sup> state (Fig. 4a), as previously reported for the resting WT FdhAB, with  
212 a closed disulfide bond<sup>14</sup>. However, when reduced by excess formate, the C872A variant is much more rapidly  
213 inactivated than the resting form of the enzyme, showing a similar behaviour to the DTT-activated WT protein. Long-  
214 term storage in aerobic conditions of the oxidized C872A variant reveals less oxygen tolerance than that of the as-

215 isolated oxidized WT FdhAB with a closed disulfide bond (Supplementary Fig. 3). These results indicate that two factors  
216 influence the stability of the enzyme to O<sub>2</sub>: the redox state of the metal and to a less extent the state of the disulfide  
217 bond. The former plays a stronger role, with the enzyme being rapidly inactivated when it is in the reduced W<sup>IV</sup> state,  
218 but still much faster when the disulfide bond is open than when it is closed (Fig. 4a). Incubation with DTT of formate-  
219 reduced O<sub>2</sub>-exposed WT protein does not lead to recovery of activity, confirming that the loss of activity is not due to  
220 spontaneous reformation of the disulfide bond with O<sub>2</sub>.

221 Chronoamperometry was used to kinetically characterize the O<sub>2</sub> inactivation process of the formate-reduced  
222 protein during active turnover, by measuring the catalytic current in the presence of formate, which is proportional to  
223 the enzyme turnover frequency<sup>34</sup>. Notably, no current was detected for the aerobically purified oxidized WT FdhAB,  
224 consistent with the very low to no activity of the resting state with a closed disulfide bond. The rate of inactivation by  
225 O<sub>2</sub> was determined by measuring the catalytic formate oxidation current over time after injecting an aliquot of O<sub>2</sub>-  
226 saturated buffer and analysing the subsequent decrease in activity. The O<sub>2</sub> inactivation rates of aerobically purified  
227 but DTT-activated WT FdhAB, anaerobically purified WT FdhAB, and C872A variant revealed no significant differences  
228 between the three active enzymes (Fig. 4b and 4c, and Supplementary Data 3). The similar inactivation kinetics indicate  
229 that the same active state, corresponding to the conformation with the open disulfide bond, is obtained by reducing  
230 the C872-C845 bond with DTT, by isolating the enzyme anaerobically or by preventing the disulfide bond formation in  
231 the C872A variant. The inactivation is consistent with a bimolecular irreversible reaction with O<sub>2</sub> and is not reverted  
232 by applying a low redox potential (E=-670 mV vs SHE) or when the O<sub>2</sub> concentration decreases, suggesting the  
233 requirement of protein factors for activation/inactivation.

234  
235 **The C845-C872 redox switch is reversible *in vivo*.** The formation and cleavage of disulfide bonds is usually catalyzed  
236 by dedicated protein factors<sup>35</sup>. To try to assess whether the C845-C872 bond is reversibly formed *in vivo*, we performed  
237 experiments with whole cells of *D. vulgaris* over-expressing WT FdhAB. These experiments showed that the FdhAB  
238 activity of cells in anaerobic conditions is not dependent on DTT (Fig. 5a), suggesting that the enzyme is in the active  
239 state without the C845-C872 bond. Upon O<sub>2</sub> exposure for 7 min, the activity is lost but can be partially recovered by  
240 DTT, suggesting that the enzyme was converted to the resting state, with a closed disulfide bond, upon O<sub>2</sub> exposure.  
241 Incomplete recovery of the activity is expected due to some oxidative damage to the active site. Switching to bubbling  
242 with N<sub>2</sub> for another 10 min led to substantial recovery of the enzyme activity, suggesting conversion of the enzyme to  
243 the open active state. Addition of DTT further increased the activity, suggesting that part of the enzyme was still in the  
244 resting state. In the control condition, where the cells remained under O<sub>2</sub>, no activity was recovered without the  
245 addition of DTT. These results support the proposal that the C845-C872 disulfide bond is formed reversibly *in vivo* by  
246 dedicated enzymes in response to O<sub>2</sub> exposure and acts as a redox switch to control the catalysis of FdhAB (Fig. 5b).  
247 More detailed experiments will be required to fully clarify the process and the proteins involved.

248 A Blast search of the NCBI Protein database with *D. vulgaris* H FdhA identified 94 FDHs containing the double  
249 cysteine motif for this redox switch, in bacteria from the orders Desulfovibrionales, Desulfobacterales,



250 Desulfunomonadales, Syntrophobacterales and Nitrospirales (Supplementary Table 3). All these enzymes are  
251 periplasmic, 92% are Sec-containing FDHs and 70% are heterodimeric FdhAB-like enzymes.  
252

## 253 Discussion

254 The strong oxygen sensitivity of metal-dependent, and particularly W-dependent, FDHs is a major drawback  
255 for their catalytic application in the context of CO<sub>2</sub> reduction. Some metal-dependent FDHs present a degree of oxygen  
256 tolerance for the formate oxidation reaction, but are characterized by low CO<sub>2</sub> reduction activities, which limits their  
257 application<sup>36–38</sup>. Here we propose a mechanism, present in some metal-dependent FDHs from anaerobic bacteria,  
258 allowing partial protection from oxidative inactivation, based on the presence of an allosteric disulfide bond, which  
259 needs to be reduced for the enzyme to be active. Disulfide bonds in proteins were classically considered to be involved  
260 in stabilizing protein structures or having a functional role as in thiol-disulfide oxidoreductases<sup>39</sup>. However, allosteric  
261 disulfide bonds have come to be recognized as an important post-translational mechanism for controlling protein  
262 function through the triggering of conformational changes upon cleavage/formation of this bond<sup>35</sup>. Such allosteric  
263 disulfides have been identified from viruses to mammals, and many are present in extracellular human proteins and  
264 are important drug targets<sup>35</sup>. Recently, a new type of allosteric bond was also described involving a lysine and a  
265 cysteine through a nitrogen–oxygen–sulfur (NOS) bridge, which is also widespread in all domains of life<sup>40,41</sup>. Proteins  
266 with allosteric disulfides have been implicated in ligand binding, substrate hydrolysis, proteolysis, or oligomerization,  
267 but, to our knowledge, not in oxidoreductases.

268 By first oxidizing the sulfide-rich crude cell extract, we could isolate FdhAB aerobically in a stable, but almost  
269 inactive resting state, which has a closed disulfide bond and withstands O<sub>2</sub> exposure for prolonged periods of time, in  
270 the absence of reductant, allowing facile manipulation of the enzyme. This is a major advantage for catalytic CO<sub>2</sub>  
271 reduction devices as the enzyme can be easily turned on *in situ* when desired<sup>21–25</sup>. FdhAB inactivation by O<sub>2</sub> is mainly  
272 observed once W is reduced and is much faster when the disulfide bond is open than when it is closed. This suggests  
273 that formate and/or O<sub>2</sub> access to the active site is faster when this bond is open. The faster inactivation of the active  
274 form, mimicked by the C872A variant, involves the partial loss of the catalytically essential sulfido ligand, as shown in  
275 the aerobic C872A structure where it has only 56% occupancy. The sulfido ligand is more quickly displaced in the  
276 reduced W<sup>IV</sup>-SH state and at higher pH<sup>42</sup>, and its loss may be associated with superoxide production by the reduced  
277 enzyme upon contact with O<sub>2</sub><sup>43</sup>.

278 Besides the absence or very low activity of the FdhAB resting state, its pronounced decrease in formate affinity  
279 is of high physiological relevance. Formate concentrations in anoxic environments are usually in the low micromolar  
280 range (~20 μM), allowing syntrophic interactions based on formate (and H<sub>2</sub>) cycling<sup>44,45</sup>. Thus, when in the closed  
281 resting state, FdhAB is not likely to be reduced by formate *in vivo*, which leaves its active site in the O<sub>2</sub>-insensitive W<sup>VI</sup>  
282 state. This suggests that the redox switch mechanism evolved to allow protection of FdhAB against formate-induced  
283 reduction during periods of oxidative stress, by lowering formate affinity and preventing enzyme reduction and  
284 subsequent inactivation. Thus, the main role of forming the disulfide bond is probably not direct protection against  
285 oxygen, but more likely to prevent reduction of the enzyme by physiological formate concentrations to avoid

286 formation of the reduced O<sub>2</sub>-sensitive state. Once anoxic conditions are restored, the C845-C872 bond is opened,  
287 triggering structural changes that are crucial to allow CO<sub>2</sub> reduction and that drastically increase the affinity for  
288 formate and the catalytic efficiency of its oxidation. These changes were identified through the crystal structures of  
289 the C872A variant, which represent the structure of FdhAB in the activated state. These structures reveal the active  
290 conformation of the enzyme as well as the sequence of conformational changes involved in the redox switch  
291 mechanism to originate the catalytically competent state. These changes are especially relevant close to the active  
292 site, where M405 seems to have a prominent role in positioning Sec192 and maintaining the necessary active site  
293 geometry for efficient catalysis. Interestingly, a close interaction between L410 and C196, equivalent to M405 and  
294 U192 in FdhAB, was also identified in the *E. coli* Fdh-N active site using QM/MM geometry optimizations<sup>46</sup>.

295 The double cysteine motif is conserved among FDHs from other anaerobes, namely many sulfate-reducing  
296 organisms that live in environments transiently exposed to O<sub>2</sub><sup>47-50</sup>. The possibility of forming this disulfide bond in  
297 response to the redox conditions is likely an evolutionary adaptation<sup>51,52</sup> that constitutes a major advantage *in vivo* by  
298 protecting the enzyme and allowing a fast transition to the active state, without the need for *de novo* protein synthesis.  
299 In fact, sulfate reducing bacteria are notorious for having several mechanisms to detoxify O<sub>2</sub><sup>53-55</sup>, and also for having  
300 evolved ingenious systems for protecting sensitive enzymes from oxidative stress, based on formation of resting states  
301 that are resistant to damage but can be quickly reactivated when O<sub>2</sub> is removed. Examples are PFOR (pyruvate-  
302 ferredoxin oxidoreductase), which presents a protein extension that can protect an O<sub>2</sub>-sensitive iron-sulfur cluster and  
303 is locked in this position by formation of a disulfide bond<sup>56</sup>, and the [FeFe] and [NiFeSe] hydrogenases where a transient  
304 sulfur ligand at the active site protects them from oxidative damage<sup>57-59</sup>. An alternative strategy is present in the CO  
305 dehydrogenase where the typical [4Fe-4S] cluster close to the surface is substituted by an oxygen-resistant [2Fe-2S]  
306 cluster<sup>60</sup>.

307 In conclusion, we identified a redox switch mechanism present in the *D. vulgaris* H FdhAB that forms the basis  
308 for its remarkable oxygen tolerance and allows its successful application in light or electricity-driven CO<sub>2</sub> reduction  
309 systems<sup>21-25</sup>. This mechanism is based on an allosteric disulfide bond that controls enzyme activity and formate affinity,  
310 and physiologically prevents enzyme reduction and subsequent O<sub>2</sub> sensitivity. The redox switch is present in other  
311 FDHs from anaerobic bacteria, most typically in highly active as well as highly O<sub>2</sub>-sensitive W- and Sec-containing  
312 heterodimeric FDHs and is likely a key evolutionary advantage in the aerotolerance of these organisms.

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### 331 **Author Contributions**

332 I.A.C.P. and A.R.O. conceived and designed biochemical experiments and sequence analysis. A.R.O. performed  
333 molecular biology experiments, protein purification, biochemical characterization, enzymatic assays, sequence  
334 analysis, EPR and electrochemical studies of WT, C845A and C872A variants and figure preparation. R. R. M. produced  
335 and characterized the M405A variant and contributed to CO<sub>2</sub> reduction assays and figure preparation. C.M., M.J.R. and  
336 G.V.A. designed the crystallography experiments and analysed the crystal structures. C.M. and G.V.A. performed TSA  
337 assays, crystallized the proteins, solved and refined all structures. G.V.A. prepared all figures with crystal structures.  
338 K.K. helped with crystallization assays and first stages of refinement of the 8CM4 and 8CM5 structures. V.F. and C.L.  
339 designed electrochemical experiments, performed by V.F. and A.R.O.. B.G. designed and analyzed EPR experiments,  
340 performed by B.G. and A.R.O.. I.A.C.P., R.R.M. and N.P. designed in vivo experiments, performed by N. P. and R.R.M..  
341 I.A.C.P. and M.J.R. supervised and funded the project. A.R.O., I.A.C.P., M.J.R., C.M., G.V.A., wrote the manuscript with  
342 inputs from co-authors V.F., C.L. and B.G. All authors approved the final version of the manuscript.

### 344 **Competing interests**

345 The authors declare no competing interests.

347

348 **Table 1 | Kinetic parameters of FdhAB WT, C872A, and M405A variants<sup>a</sup>**

Condition	DTT in assay		Formate			CO <sub>2</sub>			Ref
			$K_M^a$ ( $\mu\text{M}$ )	Turnover ( $\text{s}^{-1}$ )	$k_{\text{cat}}/K_M$ ( $\text{s}^{-1}\text{mM}^{-1}$ )	$K_M^a$ ( $\mu\text{M}$ )	Turnover ( $\text{s}^{-1}$ )	$k_{\text{cat}}/K_M$ ( $\text{s}^{-1}\text{mM}^{-1}$ )	
Aerobic	+	WT	$16.9 \pm 2.8$	$1,310 \pm 50$	77,515	$324 \pm 54$	$344 \pm 41$	1,090	14
Aerobic	-		$2560 \pm 168$	$94 \pm 10$	36	-	-	-	this work
Anaerobic	-	C872A	$33.7 \pm 4.2$	$1,485 \pm 95$	44,065	$173 \pm 16$	$368 \pm 22$	2,140	this work
Anaerobic	-	M405A	$17.9 \pm 1.7$	$82.4 \pm 1.8$	4,603	$12,253 \pm 1,942$	$43 \pm 3$	3.5	this work

349 <sup>a</sup>Kinetic data were obtained in 50 mM KPi buffer pH 7.6, 2 mM benzyl viologen for formate oxidation, and 100 mM KPi  
350 buffer pH 7.1, 0.1 mM reduced methyl-viologen for CO<sub>2</sub> reduction. For determination of the affinity constants, formate  
351 concentrations ranging from 0.5  $\mu\text{M}$  to 20 mM and CO<sub>2</sub> concentrations from 18  $\mu\text{M}$  to 5.6 mM (WT and C872A) and  
352 312  $\mu\text{M}$  to 23 mM (M405A) were used. CO<sub>2</sub> affinity of M405A was evaluated at pH 6. The kinetic parameters (derived  
353 from data in Extended Data Figure 1) were calculated using GraphPad Prism 9 and the Michaelis-Menten equation.  
354 Values represent the mean of at least n=3 independent experiments  $\pm$  s.d..  
355

## 356 Figure Legends

357 **Figure 1: Enzymatic activity of FdhAB and variants.** **a.** Formate oxidation activity (green), and CO<sub>2</sub> reduction activity  
358 (blue) of WT FdhAB as-isolated and pre-treated with 15 mM dithionite prior to activity measurements, evaluated with  
359 (+) and without (-) pre-incubation with DTT. 100% of activity calculated with the standard assay (as-isolated enzyme  
360 pre-incubated with DTT). Data are based on n=2 independent experiments. Results are plotted individually as dots,  
361 and the bars represent the mean. **b.** Representation of WT FdhAB structure highlighting the redox centers and the  
362 position of the C845-C872 disulfide bond (distance in Å). **c.** Formate oxidation turnover rates of WT FdhAB (green),  
363 C845A (grey), C872A (purple) and M405A (yellow) variants, aerobically and anaerobically purified. **d.** CO<sub>2</sub> reduction  
364 turnover numbers of WT FdhAB (blue), C845A (grey), C872A (purple) and M405A (yellow) variants, aerobically and  
365 anaerobically purified. Data are based on at least n=3 independent experiments. Results are plotted individually as  
366 dots, and the bars represent the mean.

367  
368 **Figure 2: EPR spectra of W<sup>V</sup> species in WT FdhAB enzyme (black traces), C872A (blue traces) and M405A (magenta**  
369 **trace) variants.** Experimental spectrum of a) resting WT sample poised at -468 mV by reduction with dithionite; b) DTT  
370 activated WT sample poised at -395 mV by reduction with formate; c) C872A sample poised at -443 mV by reduction  
371 with formate; d) C872A sample poised at -469 mV by reduction with dithionite; e) M405A reduced by dithionite. EPR  
372 conditions: temperature 80 K; microwave power 40 mW at 9.479 GHz; modulation amplitude 1 mT at 100 kHz.

373  
374 **Figure 3: Structural changes induced by the allosteric cleavage of the C845-C872 bond.** Superposition of FdhAB WT  
375 (grey) and C872A<sub>anox</sub> structures (blue). **a)** Close-up view of the C872A mutation region. **b)** F862---K868 and S984---P993  
376 loops. Loop F862---K868 is defined in the structure of C872A<sub>anox</sub> and is displacing loop S984---P993. I992 is shown as  
377 sticks and the distances between I992 Cδ (in C872A<sub>anox</sub>) and U192 Cα, in both structures, are indicated. **c)**  
378 Conformational changes induced by the lack of the disulfide bond. Residues whose conformations are altered are  
379 shown as sticks. Black arrows indicate the allosteric mechanism linking the disulfide bond and the active site. **d)** View  
380 of the active site including U192, M405 and Q409. **e)** Close-up view of W active site and M405/U192 movement.

381  
382 **Figure 4: Effect of oxygen on catalysis by WT FdhAB and C872A variant.** **a.** Effect of O<sub>2</sub> exposure on formate oxidation  
383 activity of as-isolated WT FdhAB (grey symbols)<sup>14</sup>, WT FdhAB pre-activated with DTT (green symbols), and C872A  
384 variant (blue symbols), incubated aerobically at room temperature in buffer A (open symbols) and buffer A plus 20  
385 mM formate (full symbols). The as-isolated WT FdhAB is treated with DTT only prior to activity measurement. Data  
386 are presented as mean values ± s.d. (n = 2 or 3 assay technical replicates). **b.** Effect of O<sub>2</sub> (introduction marked by blue  
387 arrow) on formate oxidation current of the active forms of FdhAB: DTT-activated WT FdhAB (green line); anaerobically  
388 purified WT FdhAB (grey line); C872A variant (dashed blue line). **c.** Rate of O<sub>2</sub> inactivation calculated according to  
389 equation 3 (Supplementary Data 3), from the mean of n=3 independent experiments ± s.d.. Experimental conditions:  
390 [O<sub>2</sub>] = 30 μM, t = 60 s, 1 mM formate, E = 0.130 V, pH 7, electrode rotation rate = 4000 rpm. Differences are not  
391 significant (ns, p>0.05) as estimated using GraphPad prism 7, one-way ANOVA, and Tukey's multiple comparisons test.

392  
393 **Figure 5: Reversibility of the FdhAB redox switch.** **a)** Formate oxidation activity of *D. vulgaris* H cells expressing  
394 recombinant WT FdhAB, without (green) and with (blue) DTT activation, at time zero (T0), after exposure to 7 min O<sub>2</sub>  
395 (T7-O<sub>2</sub>), and after further 10 min N<sub>2</sub> (T17-N<sub>2</sub>) or continued 17 min O<sub>2</sub> (T17-O<sub>2</sub>). All data are based on at least n=3  
396 independent experiments. Results are plotted individually as dots, and the bars represent the mean. The expected  
397 status of the C845-C872 disulfide bond is represented. **b)** Scheme of the physiological FdhAB cycle. Upon aerobic  
398 treatment of the crude extract and isolation the enzyme is in a resting state with a closed disulfide bond, thus  
399 preventing reduction by physiological concentrations of formate. Upon cleavage of the C845-C872 bond the enzyme  
400 is activated and can enter the catalytic cycle (grey box). The reduced W<sup>IV</sup> state is very sensitive to O<sub>2</sub>-induced  
401 inactivation.

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564

## 565 **Methods**

566 **Site-Directed Mutagenesis, Expression, and Protein Purification.** FdhAB variants were produced by site-directed  
567 mutagenesis of *fdhA* gene in the pRec-FdhAB-Strep expression plasmid<sup>14</sup> using the NZYMutagenesis kit. The primers  
568 used are presented in Supplementary Table 5. Correct mutations were confirmed by sequencing by Eurofins Genomic,  
569 Germany. Plasmid incorporation was performed by electroporation and cells were grown as reported for the *D.*  
570 *vulgaris* H  $\Delta$ *fdhAB* deletion strain<sup>14,20,61</sup>. For aerobic purification of FdhAB and its variants, the crude soluble extract  
571 was oxidized in air until the redox potential was stable (from an average of -270 mV to +155 mV, vs SHE). The affinity  
572 purification was performed following the protocol reported in<sup>14</sup>. For anaerobic purifications, all the purification steps  
573 were employed using N<sub>2</sub>-flushed buffers. The disruption was equally done using the French pressure cell, and for that,  
574 a tube was adapted at the exit of the cell to connect with a rubber stopper closed glass shot through a needle. This  
575 way, a close circuit was created minimizing contact with air. After ultracentrifugation, the soluble fraction was flushed  
576 with N<sub>2</sub> to remove the remaining sulfide and 2 mM DTT was added. Affinity purification was performed inside a COY  
577 Anaerobic chamber with an atmosphere of 2% H<sub>2</sub>/98% N<sub>2</sub>. Unless otherwise stated, FdhAB was aerobically purified,  
578 while C872A and M405A were typically anaerobically purified. The buffer of eluted samples was exchanged to aerobic  
579 or anaerobic 20 mM Tris-HCl pH 7.6, 10% (v/v) glycerol and 10 mM NaNO<sub>3</sub> (Buffer A). Protein concentration was  
580 determined based on  $\epsilon_{410\text{nm}} = 43.45 \text{ mM}^{-1}\text{cm}^{-1}$ <sup>14</sup>. Purity of samples was judged by 12% SDS-polyacrylamide gel.

581

582 **Solution Activity Assays.** Routine solution enzymatic assays were done as previously reported<sup>14</sup>, with and without DTT  
583 pre-treatment, using a UV-1800 Shimadzu spectrophotometer, inside a COY Anaerobic chamber with an atmosphere  
584 of 2% H<sub>2</sub>/98% N<sub>2</sub>. A final concentration of 1.4 nM of enzyme was used for WT, C872A and C845A FdhAB variants, while  
585 for M405A a final concentration of 14 nM was used.

586 The effect of dithionite on enzyme activation was evaluated by incubating WT FdhAB for 5 minutes with 15  
587 mM dithionite, corresponding to 100-fold relative to the enzyme. Excess dithionite was washed away using anaerobic  
588 buffer A and a 50- kDa cut-off centrifugal filter unit, and enzyme concentration was determined again, and the same  
589 1.4 nM FdhAB were used in the activity assays, with and without DTT pre-treatment. Similarly, for the pre-activation  
590 with DTT, FdhAB was incubated for 5 minutes with 50 mM DTT, and the excess was removed as described above. The  
591 influence of pH on FDH activity was evaluated using a buffer mix of glycine, K<sub>2</sub>HPO<sub>4</sub>, citric acid and Tris, 25 mM each.  
592 The pH was adjusted using HCl or KOH.

593 For the determination of the affinity constants for formate, the activity of pure enzyme samples was measured  
594 at substrate concentrations ranging from 0.5  $\mu$ M to 20 mM sodium formate, in 50 mM KPi buffer at the optimum pH  
595 of 7.6. In the case of affinity to CO<sub>2</sub>, 125  $\mu$ M to 50 mM of sodium bicarbonate were used, in 100 mM KPi at pH 7.1. The  
596 bicarbonate stock solutions (in 100 mM KPi, pH 7.1) were kept in fully filled flasks and the enzymatic reactions were  
597 carried out with a phase of mineral oil on top of the solution, to avoid headspace and minimise CO<sub>2</sub> loss to the gas  
598 phase. This revealed a lower K<sub>M</sub> for CO<sub>2</sub> for the WT FdhAB than previously determined<sup>14</sup>. The pH of the enzymatic

599 reactions was confirmed for each point. CO<sub>2</sub> affinity of M405A was evaluated at pH 6. Activities with CO<sub>2</sub>-saturated  
600 buffer were identical, as expected from the relatively fast CO<sub>2</sub>-HCO<sub>3</sub><sup>-</sup> equilibrium<sup>62</sup>.

601  
602 **Whole cell assays.** *D. vulgaris* H cells expressing recombinant WT FdhAB were grown anaerobically, as previously  
603 reported<sup>14</sup>. The formate oxidation activity of fresh whole cells in growth medium (OD<sub>600</sub> = 0.3, 50 μl) was measured  
604 anaerobically, with and without 1 mM DTT in 1 ml of 50 mM KPi pH 7.6, 20 mM sodium formate, 15 mM  
605 ethylenediaminetetraacetic acid (EDTA) and 1 mM methyl viologen. Activities with DTT involved a 2.5 min pre-  
606 activation with 50 mM DTT. Reduction of methyl viologen was followed spectrometrically at 578 nm ( $\epsilon_{578\text{ nm}}(\text{MV}^+) =$   
607  $9.7\text{ mM}^{-1}\text{ cm}^{-1}$ ). Cell cultures were then exposed to air bubbling for 7 min, with stirring. Part of the cultures was shifted  
608 to N<sub>2</sub> bubbling for 10 min, while the remaining cultured remained exposed to air bubbling. It was previously shown  
609 that such oxidative stress induces a stress response, but does not lead to generalised cell death<sup>63-66</sup>. Aliquots were  
610 periodically collected and evaluated for formate oxidation activity with and without DTT activation, as described for  
611 the initial activity. The *D. vulgaris* H  $\Delta\text{fdhAB}$  deletion strain grown with tungsten showed no activity for formate  
612 oxidation, due to the low expression of the other two FDHs present in the genome<sup>19</sup>.

613  
614 **Thermal Shift Assays.** The melting temperature of C872A and C845A was determined using the Applied Biosystems  
615 Protein Shift Dye Kit: 2 mg/mL of pure samples in buffer A were mixed with the dye (2-fold) and the melting curve  
616 recorded from 25 to 99°C, on the QuantStudio 7 Flex Real-time PCR system from Applied Biosystems.

617  
618 **Electrochemical Methods.** All procedures were performed inside a Jacomex glove box with an atmosphere of N<sub>2</sub> (O<sub>2</sub> <  
619 4 ppm). Aerobically isolated FdhAB was pre-treated with DTT by mixing 1 μL of 74 μM enzyme with 1 μL of 10 mM  
620 DTT, for 10 minutes. The mixture was diluted to 7.4 μM using buffer E (100 mM NaCl, 5 mM MES, 5 mM CHES, 5 mM  
621 HEPES, 5 mM TAPS, 5mM sodium acetate, pH 7.0). Anaerobically purified WT FdhAB, and C872A and C845A variants  
622 were not treated with DTT and were similarly diluted with buffer E. To produce the electroactive films the different  
623 enzyme preparations were co-absorbed on pyrolytic graphite edge rotating disc working electrode (PGE-RDE, 2.5 mm  
624 diameter), with polymyxin B sulfate (Sigma-Aldrich). For that 0.5 μL of 6 mg/mL polymyxin were deposited in PGE-RDE  
625 and left to dry, and then 0.5 μL of 7.4 μM enzyme solution were added and the electrode was used immediately before  
626 the enzyme solution dried, as described in<sup>62</sup>. The electrochemical measurements were performed in a standard three-  
627 electrode cell, a platinum wire was used as the counter electrode, and saturated calomel electrode as the reference  
628 electrode. Measurements were made using the Autolab PGSTAT128N potentiostat (Metrohm, The Netherlands) with  
629 the software GPES. The rotation of the working electrode to around 4000 rpm was controlled using an Autolab RDE 2  
630 electrode rotator (Metrohm, The Netherlands). Chronoamperometric measurements were done in the presence of 1  
631 mM formate in buffer E, and the potential poised to  $E = 0.142\text{ V}$  vs standard hydrogen electrode (SHE). Additions of 30

632  $\mu\text{M O}_2$  were performed by injecting in the cell 50  $\mu\text{L}$  of  $\text{O}_2$ -saturated  $\text{H}_2\text{O}$ . The data was analyzed using the open-source  
633 program QSoas<sup>67</sup> applying the following commands:

634 apply-formula  $y=\log(y)$

635 filter-fft /derive=1

636 fit-exponential-decay

637 QSoas<sup>67</sup> is a free software available at <https://bip.cnrs.fr/groups/bip06/software/>

638

639 **EPR Spectroscopy.** The reduction of C872A variant (39  $\mu\text{M}$ ) prepared in 50 mM MOPS pH 7.6, 10% (v/v) glycerol was  
640 done inside a glove box (Jacomex), at room temperature. The redox potential was measured in the titration cell with  
641 a combined Pt-Ag/AgCl/KCl (3M) microelectrode. Methylene blue, indigo disulfonate, phenosafranine, methyl red, and  
642 methyl viologen were used as mediators to a final concentration of 10  $\mu\text{M}$ . Successive additions of either sodium  
643 formate or sodium dithionite were done. After potential stabilization, a sample was collected to an EPR tube and  
644 frozen immediately in the glove box. For the M405A variant, the reduction was performed inside the glove box by  
645 adding directly a small volume of sodium formate or sodium dithionite to the EPR tubes.

646 EPR analysis was done on a Bruker ELEXSYS E500 spectrometer equipped with an ER41002ST standard  
647 rectangular Bruker EPR cavity fitted to an Oxford Instruments ESR 900 helium flow cryostat. Double integration of EPR  
648 spectra recorded in non-saturating conditions was done for spin intensity measurements, comparing with 1 mM  
649 Cu(II)EDTA standard. EPR spectrum simulations were performed with EasySpin.

650

### 651 **Crystallization, Data Collection, Structure Solution, and Refinement.**

652 The crystal structures reported in this work were obtained from C872A samples purified anaerobically and  
653 M405A purified in the presence of oxygen<sup>14</sup>. All crystals were obtained at 20°C using hanging-drop vapor diffusion  
654 method, drops of 2  $\mu\text{L}$  (1:1, protein:precipitant ratio) in 24 well plates (24 well XRL plate Molecular Dimensions). The  
655 concentration of variant C872A was 18.2 mg/mL and the crystals appeared in three different conditions; C872A\_ox  
656 (aerobic conditions): 20 % PEG 3350 (w/v), 0.1 M Tris-HCl pH 8.5 and 0.2 M  $\text{CaCl}_2$  (crystals appeared within 2 days);  
657 C872A\_anox\* (anaerobic conditions): cocrystallized with 10 mM of azide in 22% PEG 3350 (w/v), 0.1 M Tris-HCl pH 8.5  
658 and 0.2 M  $\text{CaCl}_2$  (crystals appeared within 2 days); C872A\_anox (anaerobic conditions): 32% PEG 3350, 0.1 M Tris HCl  
659 pH 8.0, 1 M LiCl (the condition previously optimized for the wild type crystals<sup>14</sup>) with 0.2  $\mu\text{L}$  of a dilution 1:100 from a  
660 stock of microseeds from FdhAB wild type, these crystals were finally soaked for 30 min with 10 mM of sodium  
661 formamide (crystals appeared within 30 days). All the anaerobic experiments were performed in an anaerobic  
662 chamber under an argon atmosphere at <0.1 ppm of oxygen, and all the solutions were previously degassed and stored  
663 in the anerobic chamber. Crystals of M405A (at 11.5 mg/mL) were obtained in 30% PEG 3350, 0.1 M Tris HCl pH 8.0

664 and 1 M LiCl in aerobic conditions. All crystals were transferred into a cryoprotectant solution consisting of the  
665 precipitant solution supplemented with 20% (v/v) glycerol, and then flash cooled in liquid nitrogen.

666 X-ray diffraction experiments were performed on ALBA (XALOC beamline)<sup>68</sup> and ESRF (ID23-1, ID30A-3 and  
667 ID30B)<sup>69–71</sup> synchrotrons and the data were processed with the programs XDS<sup>72</sup> and Aimless<sup>73</sup> or autoPROC<sup>74</sup> and  
668 STARANISO<sup>75</sup>. The structures were solved by molecular replacement with Phaser<sup>76</sup> from the CCP4 suite<sup>77</sup>, using as  
669 search model the previously published as-isolated structure (PDB ID: 6SDR) to the C872A<sub>ox</sub> and M405A variants,  
670 C872A<sub>ox</sub> for the C872A<sub>anox</sub>\*, and C872A<sub>anox</sub>\* for the C872A<sub>anox</sub>. The models were refined with iterative cycles  
671 of manual model building with Coot<sup>78</sup> and refinement with REFMAC5<sup>79</sup>. Data processing and refinement statistics are  
672 presented in Supplementary Table 1.

673  
674 **Sequence analysis.** The amino acid sequence of *D. vulgaris* H FdhA (Uniprot Q72EJ1, Locus tag DVU0587, NCBI  
675 Reference Sequence: WP\_010937890.1) was used as query in the Protein-Protein Basic Local Alignment Search Tool  
676 (BLASTp), against the RefSeq Selected proteins database (Feb 2021). Multiple sequence alignment was performed  
677 using Clustal Omega<sup>80</sup> and analyzed using Jalview software<sup>81</sup> and *D. vulgaris* H FdhA as reference. The alignment was  
678 manually curated namely in the adjacent regions to positions 845 and 872.

679 The genome of bacteria identified as having a CC-FDH were selected on Integrated Microbial Genomes &  
680 Microbiomes (IMG/M), but only 77 from the 85 were available. A blast using DVU0587 as query was performed against  
681 the selected genomes and the locus tag of the respective FDH sequences containing the conserved cysteines (CC-FDH)  
682 were identified. Then the CC-FDH operons were analyzed to identify the complexity of FDH composition, named as AB  
683 for dimeric protein (catalytic and electron transfer subunit), ABC for trimeric protein containing an additional  
684 cytochrome *c*<sub>3</sub> subunit and ABG for trimeric protein containing a membrane-bound cytochrome *b* ( $\lambda$  subunit). The  
685 presence of a Tat signal peptide was predicted using SignalP - 5.0 software<sup>82</sup>.

## 686 687 **Data availability**

688 The data that support the findings of this study is available within the main text and its Supplementary Information  
689 file. Source data is provided as Source Data files. The atomic coordinates and structure factors for the *D. vulgaris* H  
690 C872A variant structures have been deposited in the Protein Data Bank under accession codes [8CM4](#), [8CM5](#), [8CM6](#)  
691 and [8CM7](#).

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Figure 1

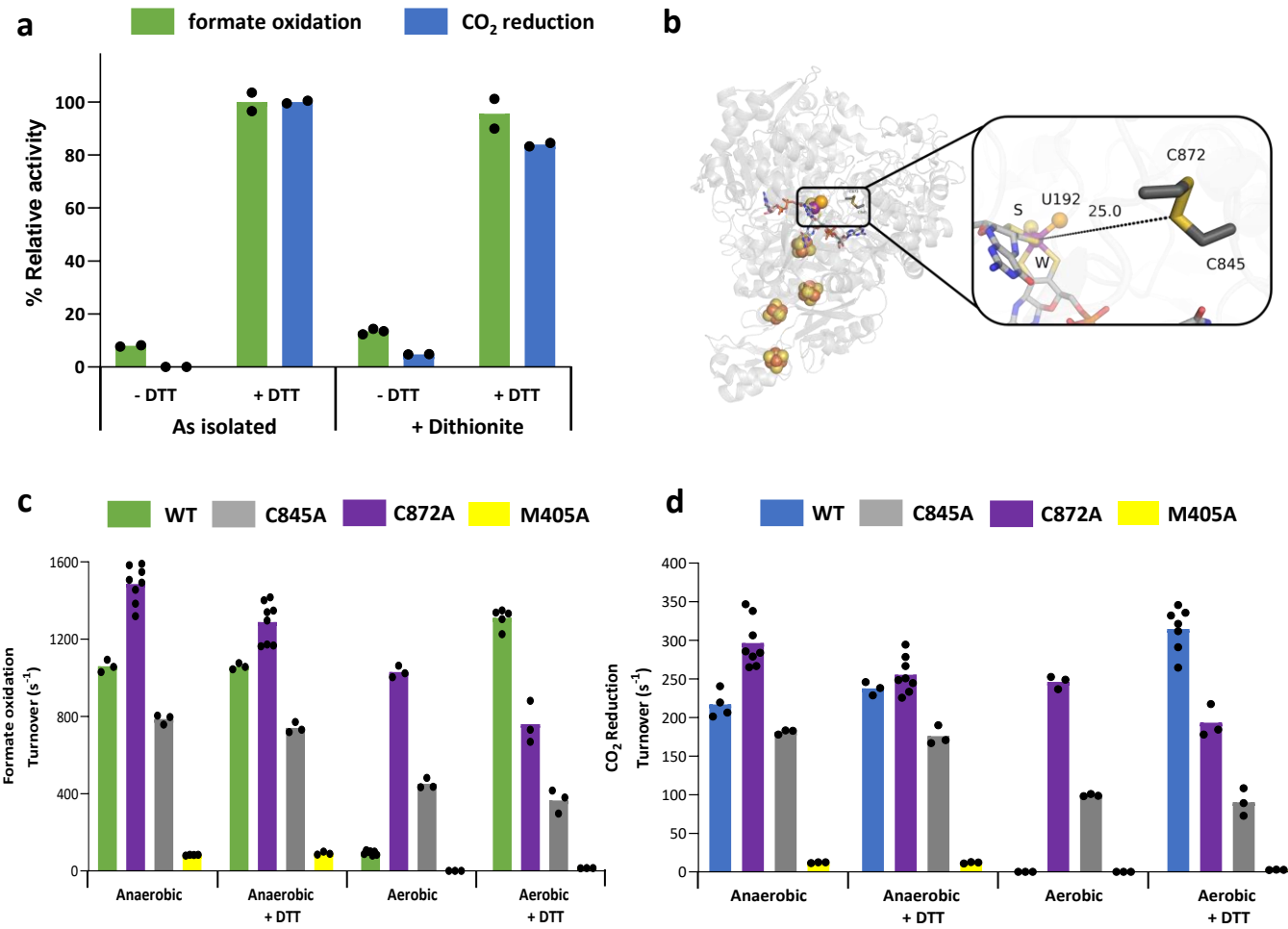


Figure 2

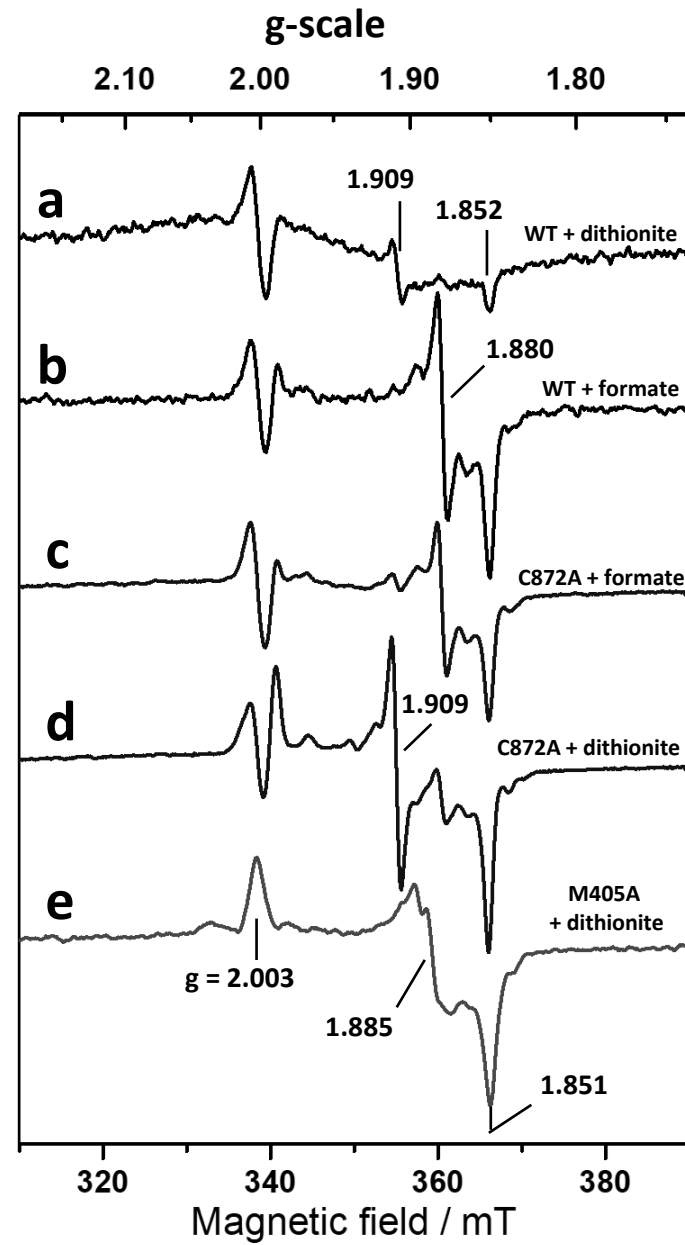


Figure 3

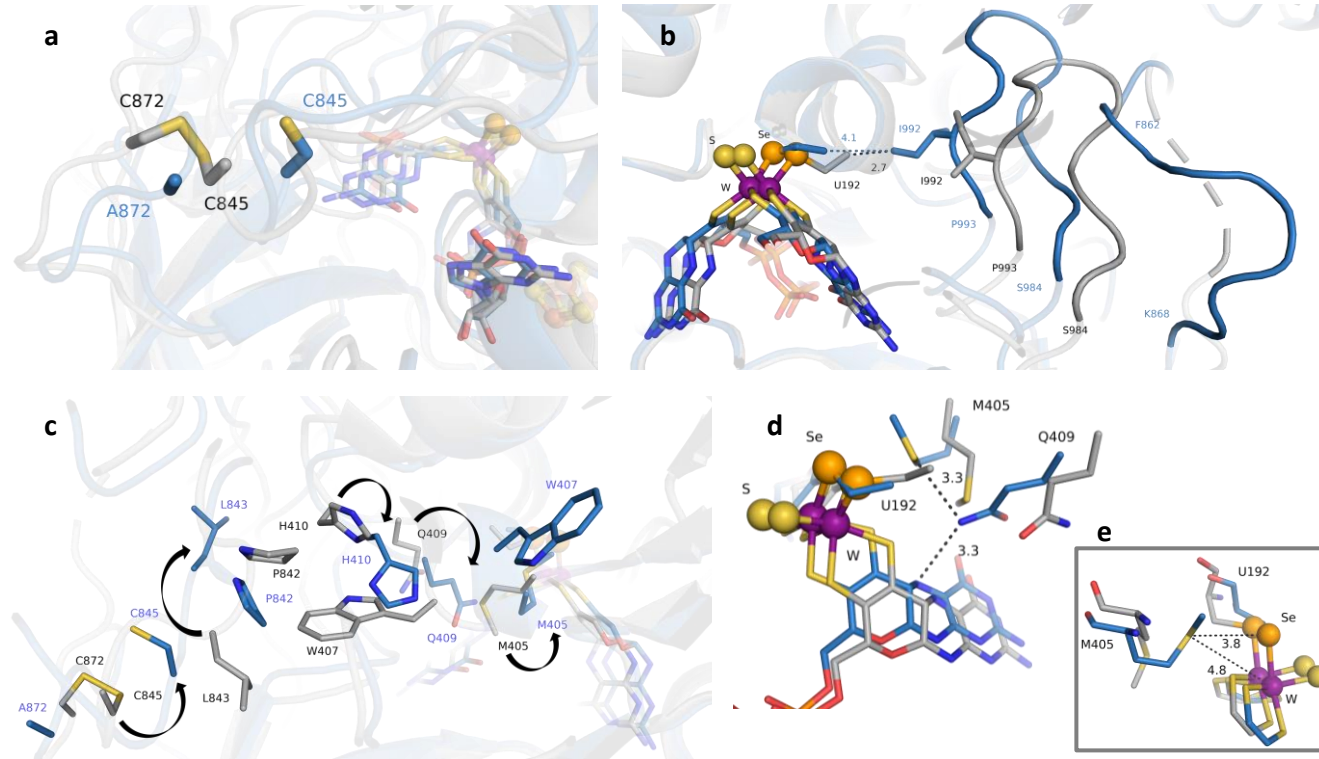


Figure 4

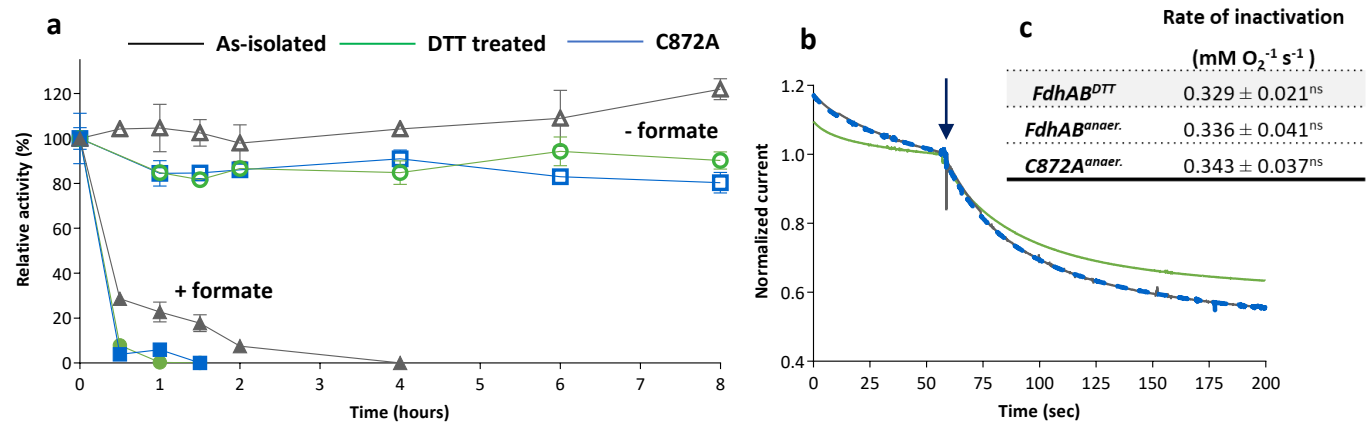


Figure 5

