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Toward the Mechanistic Understanding of Enzymatic CO₂ Reduction

Ana Rita Oliveira, Cristiano Mota, Cláudia Mourato, Renato M. Domingos, Marino F. A. Santos, Diana Gesto, Bruno Guigliarelli, Teresa Santos-Silva, Maria João Romão,* and Inês A. Cardoso Pereira*

ABSTRACT: Reducing CO₂ is a challenging chemical transformation that biology solves easily, with high efficiency and specificity. In particular, formate dehydrogenases are of great interest since they reduce CO₂ to formate, a valuable chemical fuel and hydrogen storage compound. The metal-dependent formate dehydrogenases of prokaryotes can show high activity for CO₂ reduction. Here, we report an expression system to produce recombinant W/Sec-FdhAB from Desulfovibrio vulgaris Hildenborough fully loaded with cofactors, its catalytic characterization and crystal structures in oxidized and reduced states. The enzyme has very high activity for CO₂ reduction and displays remarkable oxygen stability. The crystal structure of the formate-reduced enzyme shows Sec still coordinating the tungsten, supporting a mechanism of stable metal coordination during catalysis. Comparison of the oxidized and reduced structures shows significant changes close to the active site. The DvFdhAB is an excellent model for studying catalytic CO₂ reduction and probing the mechanism of this conversion.

KEYWORDS: formate dehydrogenase, CO₂ reduction, X-ray structure, oxygen-tolerance, tungsten, molybdopterin, moco

INTRODUCTION

Developing sustainable processes to reduce the levels of atmospheric CO₂ is one of the most urgent and difficult issues facing our society. Converting CO₂ is a chemical challenge due to its intrinsic kinetic and thermodynamic stability. Chemical conversions require high energy inputs, have low selectivity, low catalytic efficiencies, and often need rare-metal catalysts. This contrasts with the high turnovers, high specificity, and mild conditions observed in biology (through reductases, carboxylases, and lyases). Studying the biochemical strategies, Nature has developed to convert CO₂ is thus paramount to inspire development of more selective, efficient, and sustainable catalysts. CO₂ reduction to formate is the basis of the most energy-efficient biological pathway of carbon fixation, the reductive acetyl-CoA pathway, which is probably also the most primordial form of carbon fixation. Technologically, this reduction is of high interest as it allows CO₂ sequestration as a valuable commodity chemical (formate), providing a means of H₂ storage.

Formic acid is a safe liquid, nontoxic and nonflammable, with high volumetric energy density, and can easily be transported. Direct formic acid fuels cells are already a reality.

In biology, CO₂ reduction to formate is performed by formate dehydrogenases (FDHs). These enzymes are divided in two classes: the metal-free, oxygen-tolerant, NAD⁺-dependent enzymes present in plants, fungi, and some aerobic bacteria, and the molybdendum- or tungsten-dependent enzymes present in anaerobic prokaryotes. The metal-free, NAD⁺-dependent enzymes are designed for formate oxidation (CO₂ reduction from NADH is thermodynamically uphill), and are extremely slow in the reductive direction. In contrast, both the Mo- and W-dependent FDHs catalyze CO₂ reduction, even in the case of the NAD(1)-dependent, multimeric Mo-FDHs. In the metal-dependent FDHs, the active site consists of a Mo/W atom coordinated by two Molybdopterin Guanine Dinucleotides (MGD), one selenocysteine (Sec) or cysteine residue and one terminal sulfido (=S/−SH) ligand. The sulfido ligand is introduced by a dedicated chaperone and is essential for activity.

Despite considerable studies, the mechanism used by FDHs to oxidize formate or reduce CO₂ is still unclear. Several proposals have been made, and the topic has raised considerable debate. Critical issues still under discussion are (i) whether the Sec/Cys ligand dissociates from the metal during catalysis and is replaced by formate, or if metal coordination is maintained during catalysis; and (ii) whether oxidation of formate involves hydride transfer to the metal or sulfido group or C₆ proton abstraction by a protein residue. A number of experimental and computational tools have been explored to try to elucidate this mechanism, namely X-ray crystallography.

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ion spectroscopy, theoretical calculations, and inhibition studies. However, the results are not always conclusive due to the difficulty in confidently assigning the observations to relevant catalytic states, as summed by the intrinsic instabilities of the proteins, which may be present in inactivated states. There is, thus, an urgent need for detailed structural information on FDHs in different catalytic/redox states.

When focusing on CO₂ reduction to formate it seems logical to investigate FDHs that physiologically perform this function, rather than formate oxidation. This is the case of enzymes present in acetogens, which grow from converting CO₂ to acetate, in syntrophs, which produce formate and H₂ as vehicles for interspecies electron transfer, and finally in the closely related sulfate reducing bacteria, which in the absence of sulfate also turn to a syntrophic lifestyle. In fact, the enzymes with the highest CO₂-reducing activities are all derived from these organisms, and include the hydrogen-dependent CO₂ reductases (HDCR) from the acetogens Acidobacterium woodii and Thermoanaerobacterium kivui, the W-FDH from the syntroph Syntrophobacter fumaroxidans, and the Mo-FDH from the sulfate reducer Desulfovibrio desulfuricans.

Here, we report a novel expression system for production of a W/Sec-FDH from Desulfovibrio vulgaris Hildenborough, DvFdhAB, and its catalytic and structural characterization. The DvFdhAB is responsible for CO₂ reduction in D. vulgaris and is the main FDH expressed in this organism if tungsten is available, revealing its physiological preference over the Mo-FDH from the related sulfate reducing bacteria, which in the absence of sulfate produce pure DvFdhAB (Figure S3). Identical reduction levels were obtained with formate as with dithionite (Figure S4), indicating that the enzyme is fully loaded with the tungsten-sulfido group (W=≡S²⁻≡SH), essential for reduction by formate.

Metal-dependent FDHs often require reductive activation to display maximal activity. In contrast to the D. desulfuricans Mo/Sec-FdhABC, a 30 min incubation with formate was not enough to fully activate DvFdhAB, as a significant lag phase was still observed (Figure S5). Preactivation for 30 min with both formate and DTT abolished this lag phase. Furthermore, just 5 min preincubation of DvFdhAB with 50 mM DTT (or TCEP) was enough to observe maximum initial activities, indicating full activation.

Enzyme Kinetics. DvFdhAB oxidizes formate with a turnover rate of 1310 ± 50 s⁻¹ (Table 1). In contrast to a previous report where DTT preactivation was not used, the DvFdhAB is in fact highly active in catalyzing CO₂ reduction with a turnover of 315 ± 28 s⁻¹. This robust CO₂-reducing activity agrees with the fact that in vivo this enzyme is responsible for formate production, when W is available. The activity of native DvFdhAB was reevaluated in the new assay conditions, showing similar rates to the recombinant version: a turnover of 1104 ± 62 s⁻¹ for formate oxidation, and of 304 ± 41 s⁻¹ for CO₂ reduction. These high catalytic activities place the W/Sec-DvFdhAB among the most active CO₂ reductases yet reported.

The DvFdhAB pH optimum for formate oxidation is 7.6, but the activity is sustained close to 80% between pH 7 and 10 (Figure 1B). The pH optimum of the reductive reaction is 7.1, and the activity is held above 60% between pH 4.7 and 7.6. Due to the influence of carbonate on the pH (see SI text), the CO₂ concentration varies across the pH range. With 50 mM sodium bicarbonate as the CO₂ source the concentration of CO₂ (1) is only 3.6 mM at pH 7.5, and decreases drastically above that, to only 120 μM at pH 9. In addition, the ionic strength of the solution also increases at higher pHs, which may also have a detrimental effect on activity. These two factors likely explain the drastic drop in activity above pH 7. The kinetic constants were determined for both reactions at the pH optimum (Figure IC/D, Table 1). The Kₘ for formate is 16.9 ± 2.8 μM and the Kₘ for CO₂ is 6.6 ± 2.8 μM (see SI text), indicating that the enzyme is fully loaded with the tungsten-sulfido group. The Kₘ for CO₂ is 420 ± 47 μM (see SI text). The only FDH reported with higher CO₂ affinity is the D. desulfuricans Mo-FDH, with a Kₘ of 15.7 μM. However, this value was calculated by a linearized graphical method, which can introduce significant errors.

Inhibition by nitrate was tested, since this was used as a stabilizing agent. DvFdhAB retains 40% of formate oxidation and 60% of CO₂ reduction activity in the presence of 100 mM nitrate, so this is a very weak inhibitor. Since the enzyme is diluted in the activity assays (at least 100X), the effect of nitrate in these assays will be negligible. Azide is a stronger inhibitor and at 10 mM it reduced the oxidative rate to only 75%, whereas about

### Table 1. Kinetic Constants of D. vulgaris DvFdhAB

<table>
<thead>
<tr>
<th>DvFdhAB</th>
<th>HCOO⁻ oxidation</th>
<th>CO₂ reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>turnover</td>
<td>1310 ± 50 s⁻¹</td>
<td>315 ± 28 s⁻¹</td>
</tr>
<tr>
<td>Kₘ(HCOO⁻)</td>
<td>658 ± 22 Umg⁻¹</td>
<td>137 ± 12 Umg⁻¹</td>
</tr>
<tr>
<td>Kₘ(CO₂)</td>
<td>16.9 ± 2.8 μM</td>
<td>420 ± 47 μM (CO₂)</td>
</tr>
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107 RESULTS

108 Expression System. Two different strategies were tested for production of recombinant DvFdhAB: tag-labeling of the fdhA gene (DVU087), and complementation of a ΔfdhA deletion mutant with an expression vector. For the first strategy, a suicide vector was constructed based on pMO719, to directly modify fdhA in the genome (Figure S1A of the Supporting Information, SI). In the second approach, we used the D. vulgaris Hildenborough ΔfdhAB deletion strain, for which a complementation vector, pRec-FdhAB-Strep was assembled (Figure S1B). The expression of FdhA was confirmed in both systems (Figure S2). The formate oxidation activity of the complemented strain was approximately three times higher than that of the genome-modified strain. Thus, DvFdhAB was routinely produced using the complementation strategy.

122 Purification and Activation Behavior. Both W and Sec-containing enzymes are catalytically more active than their Mo and Cys counterparts, but they usually suffer from being extremely oxygen-sensitive. Remarkably, the W/Sec-DvFdhAB could be purified and handled under aerobic conditions. Nitrate and glycerol were used as stabilizing agents.

125 Nitrate is an inhibitor that helps to protect the enzyme, presumably by preventing loss of the labile sulfido group. As desired, a single affinity chromatography step was enough to produce pure DvFdhAB (Figure S3). Identical reduction levels were obtained with formate as with dithionite (Figure S4), indicating that the enzyme is fully loaded with the tungsten-sulfido group (W≡S²⁻≡SH), essential for reduction by formate.

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45% activity was sustained for CO₂ reduction. Thus, as reported for *E. coli* FdhH, the DvFdhAB is more strongly inhibited in the oxidized W(VI)−S than in the reduced W(IV)−SH state.

We could not detect any activity for nitrate reduction, in contrast to the *R. capsulatus* enzyme.

**Stability and Oxygen Tolerance.** The stability of FDHs is critical for their use in biotechnological applications. DvFdhAB is quite thermostable, with a melting temperature of 77.3 ± 1.0 °C, which is increased to 79.8 ± 0.2 °C in the presence of nitrate and glycerol. Storage of the as-isolated pure DvFdhAB at 4 °C or 19°C led to an increase in thermostability.

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**Figure 1.** Kinetic characterization of DvFdhAB. (A) Effect of pH on formate oxidation activity (20 mM formate) and (B) on CO₂ reduction activity (with 50 mM sodium bicarbonate). (C) Steady-state formate oxidation kinetics and (D) steady-state CO₂ reduction kinetics. Lines represent the direct fitting of Michaelis–Menten equation to experimental data. All assays were performed in triplicate, and the error bars show the standard deviation.

**Figure 2.** DvFdhAB structure. (Left) Structure of the heterodimer (α-subunit, red and β-subunit, blue). (Right) The metal cofactors, W(MGD)₂ and four [4Fe-4S] centers.
The DvFdhAB is a heterodimer (Figure 2) and can be divided into four (FdhA) plus three (FdhB) domains (Figure S10), following the classification of DgFdhAB. The large, catalytic α subunit harbors the [W(MGD)2, SH, Sec] cofactor and one [4Fe-4S] cluster, while the small β subunit contains three [4Fe-4S] clusters. The overall structure of the catalytic subunit is very similar to those of DgFdhAB and EcFdhN, with RMSDs of 1.30 Å for 957 β-carbons, respectively, with good geometry (Table S2).

The model was traced and refined to final crystallographic Rwork and Rfree values of 18.6% and 22.6%, respectively, with good geometry. Crystals of the formate-reduced form (DvFdhAB_red, 6SDV) diffracted beyond 1.9 Å, and the structure was solved by molecular replacement using the oxidized form as search model. The model was refined to final crystallographic Rwork and Rfree values of 15.9% and 19.7%, respectively, with good geometry (Table S2).

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The third glycerol is found in the tunnel entrance, oriented by Arg441, Thr450, and a water molecule oriented by Tyr462. Bonded by Ser194 and His457, the second glycerol is oriented by Arg441, Thr450, and a water molecule oriented by Tyr462. The third glycerol is found in the tunnel entrance, oriented by Gly207 backbone nitrogen and water molecules that make bonds with Tyr492 and Arg206. These glycerol molecules probably mimic transit formate binding sites and shed light on the formate entrance/release mechanism.

The W center is fully loaded with metal (Figure S11) according to the relative B factors for a full occupancy of all 275

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276 atoms (Table S2), and is buried 30 Å away from the protein
277 surface. In the oxidized form (DvFdhAB_ox), the metal is
278 hexacoordinated to four sulfur atoms from the two dithiolenes, a
279 selenium from Sec192 and a sulfur ligand (W−SH/−S), in a
280 distorted octahedral geometry (Figure 4a and S11). In the
281 vicinity of the metal there are two strictly conserved amino acids,
282 His193, establishing a π interaction with the Se from Sec192,
283 and Arg441, which establishes a hydrogen bond with one of the
284 glycerol molecules, and has been proposed to be responsible for
285 orienting the substrate molecule.23

286 In the formate-reduced form (DvFdhAB_red), the environ-
287 ment surrounding the active site suffers a conformational
288 rearrangement (Figure 4b,c). The Sec192 loop (Ile191-His193)
289 suffers a distortion, and the Sec192 Cα atom shifts up to 1.00 Å
290 from the position occupied in the oxidized form. Part of an
291 adjacent α-helix (Ser194-Pro198) is also displaced up to 2.25 Å
292 (Thr196ox−Thr196red, Cα−Cα distance; not shown on figure
293 for clarity). However, Sec192 remains coordinated to the metal
294 (Figure S11b). Remarkably, the catalytically relevant His193
295 moves away from the active site adopting a different rotamer,
296 and a water molecule occupies the previous position of the
297 imidazole ring, 3.18 Å away from Se, establishing a hydrogen
298 bond with Gly442 amide bond. In addition, Arg441 side chain
299 also suffers a small rearrangement. The Sec192 loop shift is
300 propagated to neighboring amino acids from different domains
301 with the consequent displacement of the adjacent hydrophobic
302 Ile191 that promotes the tilt of the side chains of Trp533,
303 Phe537, and Phe160.

304 To further confirm the W redox state in the formate-reduced
305 crystals, we performed EPR spectroscopy on a sample prepared
306 with small crystals spread in cryoprotectant solution, in parallel
307 with a dissolved sample. Due to the random orientation of
308 crystals in the polycrystal sample, the EPR spectrum can be
309 directly compared to that given by a frozen solution.51 At 15 K
310 the EPR spectra showed the [4Fe−4S]^{+}/^{2+} clusters of both
311 samples in a fully reduced state (Figure S12). These signals
312 broaden above 50 K and are no longer detected at 80 K. At this
313 temperature no slow relaxing W(V) signal is detected in either
314 sample, confirming that formate leads to full reduction of the
315 protein to the W(IV) state, which is EPR silent. Very weak
316 features are observed between g = 1.92 and 1.89 at T = 80 K but
317 they cannot be assigned to a minor proportion of W(V) species.
318
319 Finally, conformational changes are also observed for the two
320 MGDs cofactors and surrounding environment, which are more
321 pronounced for the distal MGD2. In the oxidized form, Gln890
322 is in close contact to MGD1, via a hydrogen bond to N5 of the
323 piperazine central ring (assuming a tetrahydro form, Figure 4a).
324 Additionally, Gln890 NH2 is a H-bond donor to one of the
325 dithiolene sulfur atoms and to the carboxylate of Glu443 (Figure
326 S13). In the reduced form, Gln890 adopts a new rotamer and
327 establishes a hydrogen bond to Glu443, which also adopts a
328 different conformation (Figure S13). The interaction with the
329 dithiolene sulfur atom is maintained. In contrast, the Gln890
330 C=O group is now stabilized by an interaction to a water
331 molecule present only in the reduced structure (Figures 4b and
332 S13). This water molecule is hydrogen bonded to O1A, O5′, and
333 O4′ from the dinucleotide part of MGD2. All these movements
334 seem to promote a major distortion of the ribose moiety. Gln890
335 and Glu443 are conserved in sulfate reducers, and in the
336 DvFdhAB_ox structure their positions are conserved. A similar
337 situation is observed in arsenite oxidase where a corresponding
338 Glu is also interacting with N5 of the MGD1 pterin.23 In other
339 reported FDH structures that glutamine residue is replaced by a

Figure 4. DvFdhAB active site. (a) Close-up view of the DvFdhAB_ox
340 active site and 2Fo−Fc electron density map contoured at 1σ; (b) close-
341 up view of the DvFdhAB_red active site and 2Fo−Fc electron density
342 map contoured at 1σ. (c) Superposition of the active sites of oxidized
343 (pink) and reduced (cyan) forms.

Ser (EcFdH) and by a His (EcFdH and EcNarG53). Rothery
344 and co-workers assigned a “bridging” role to this residue (Gln, Ser
345 or His) providing H-bond interconnection between both
pyranopterins. In these structures (EcFdhH ox and red) the “bridging” residue Ser587 also changes its rotamer but the smaller side chain precludes a close interaction to the MGD1 pterin.

**Formate and CO$_2$ Tunnels.** The tunnel network within the catalytic subunit was inspected with CAVER for both oxidized and reduced structures. In the oxidized form, the calculated tunnel with highest score links the positively charged funnel to the active site, assigned as the formate tunnel (Figure S14a), with a minimal diameter of 1.4 Å and a length of 13.2 Å. Strikingly, the tunnel with the highest score for the reduced structure, assigned as the CO$_2$ tunnel, is different (Figure S14b). It displays a minimal diameter of 1.8 Å and length of 39.7 Å, and also links the W active site to the protein surface. The surface of this tunnel is mainly composed by hydrophobic amino acids, and therefore it is likely to allow the access/release of nonpolar molecules such as CO$_2$. As discussed above, the imidazole ring of the conserved active site His193 is in a different position in oxidized and reduced structures. In the oxidized form its position closes the hydrophobic CO$_2$ tunnel, while in the reduced form His193 flips toward the formate tunnel opening the hydrophobic one and blocking the entrance of new formate molecules (Figure 5). Thus, the formate and CO$_2$ tunnels seem to be mutually exclusive, with His193 acting as a switch between the two.

The predicted tunnels are supported by analysis of amino acid conservation among the group of periplasmic NAD$^+$-independent FDHs (which are phylogenetically separate from the cytoplasmic ones) (Figure S15). The positively charged tunnel, for formate access/release, is composed by amino acids that present a high degree of conservation (at least 70% identity), whereas the hydrophobic tunnel presents a lower degree of conservation.

**Inhibition by iodoacetamide.** Previous reports of Sec/Cys alkylation by IAA in *E. coli* FdhH and the *R. capsulatus* Mo-Fdh were taken as indication that the Sec/Cys residue dissociates during catalysis. Since the DvFdhAB red structure indicates that Sec remains bound to W in this state, we tested the effect of iodoacetamide (IAA) alkylation on the activity.

Incubation of DvFdhAB with IAA at pH 7.6 resulted in 75% inhibition of the formate oxidation activity (Figure 6). Contrary to *E. coli* FdhH and *R. capsulatus* Mo-FDH, this inhibition was not dependent on the presence of substrate. At pH 9, the IAA inhibition is considerably lower, but also no effect of substrate or inhibitor was observed. The IAA-treated samples were analyzed by mass spectrometry to reveal which residues were alkylated (Table S3). In all samples nine Cys are always alkylated (seven Cys coordinating FeS clusters, and two near the protein surface), which explains the inhibition observed. A peptide containing the IAA-alkylated Sec192 was not detected in any of the samples. Instead, all of the samples contained a peptide where Sec is modified to dehydroalanine. This modification is frequently identified in Sec-containing peptides, resulting from the loss of selenium during the MS sample preparation and analysis. If IAA-alkylation of Sec had happened, then this would prevent loss of selenium and the alkylated peptide would be easily identified. This result suggests that the selenol ligand of DvFdhAB is not accessible for reaction with IAA, confirming the structural observations.

![Figure 5](https://example.com/fig5.png) **Figure 5.** Proposed formate and CO$_2$ tunnels. The formate tunnel is represented in green and the CO$_2$ tunnel is represented in magenta. The imidazole ring of His193 is shown in pink (oxidized form) and in cyan (reduced form).

![Figure 6](https://example.com/fig6.png) **Figure 6.** Inhibition by iodoacetamide. Formate oxidation activities after 30 min incubation without IAA (1), with 20 mM IAA (2), with 20 mM IAA and 20 mM formate (3), and with 20 mM IAA and 20 mM Nitrate (4) at pH 7.6 (A) and pH 9 (B). The activities were normalized to (1).
CO₂ reduction by FDHs can vary by orders of magnitude, and in the metal-dependent enzymes is often extremely oxygen sensitive. The metal at the active site is one important determinant for the level of this activity. Although the chemistry performed by Mo is similar to that of W, W(IV) is a stronger reductant than Mo(IV), and this is likely one of the reasons for the higher reductive catalytic efficiency of W-FDHs. Another important determinant is the presence of Sec versus Cys as metal-coordinating residue. In E. coli FdhH a Sec/Cys variant showed more than 2 orders of magnitude lower turnover than the native enzyme, and was suggested to be directly involved in the reaction. A similar effect has been observed for a Sec/Cys variant of the D. vulgaris Hildenborough [NiFeSe] hydrogenase, where the Sec was additionally shown to be essential for the oxygen tolerance of the enzyme.

The homologous expression system reported here allows production of high levels of the W/Sec-DvFdhAB fully loaded with cofactors. The simple composition of this enzyme, coupled with its high activity and high stability, enabling aerobic handling, make this enzyme an ideal model system for catalytic and mechanistic studies of CO₂ reduction. In fact, the enzyme has recently been explored for photocatalytic CO₂ reduction to CO. It has been shown that Sec/Cys alkylation by IAA may also occur even when the metal coordination is maintained. If the metal-Sec/Cys bond is broken, the enzyme is stable enough to be alkylated with IAA in DvFdhAB. Nevertheless, it should be pointed out that Sec/Cys alkylation by IAA may also occur even when metal coordination is maintained. If the metal-Sec/Cys bond is indeed labile in some enzymes, then it remains to be determined whether this has mechanistic implications or is just incidental.

Other relevant changes are observed when comparing the DvFdhAB oxidized and reduced structures. In particular, there is a pronounced movement of conserved His193, together with some aromatic residues close to the active site, which is likely to be mechanistically relevant. This is accompanied by a conformational change of the distal MGD2, which agrees with the proposal that pyranopterins are noninnocent ligands, being probably involved in redox tuning of the metal.

The contact of MGD1 with the FeS-0 center is mediated by a highly conserved lysine (Lys90 in DvFdhAB), which establishes a hydrogen bond with the exocyclic NH₂ of the pyranopterin. This conserved pathway is present both in FDHs and in related nitrate reductases. In contrast, the orientation and geometry of the distal MGD2 varies considerably among the homologous enzymes. These differences are accompanied by changes in the orientation of amino acid side chains that surround the extended cofactor. When comparing the DvFdhAB oxidized and reduced forms, the reorientation of the “bridging” Glu90 is noteworthy, suggesting a putative change in the redox state of the proximal pterin.
Gln residue to a Gly stabilized a Mo(V) state. The same was reported for E. coli Nar, where substitution of His1098 (corresponding to Gln890) by an Ala also increased the stability of the Mo(V) state. The structural changes observed in DvFdhAB together with mutagenesis data on other DMSOR family enzymes are congruent in showing the importance of the H-bond interactions bridging both pyranopterin in controlling the active site redox chemistry and catalysis. Although very high resolution (<1.5 Å) is required to assign the correct redox state to each pyranopterin, these observations raise the important point that computational predictions of enzymatic mechanisms should take into account also the putative redox role of the MGD cofactors.

**CONCLUSIONS**

In summary, we show that the recombinant DvFdhAB is a stable, highly active model system for CO2 reduction. The structures reported here suggest that Sec remains bound to the metal during the catalytic cycle but clear changes in the second coordination sphere and in pyranopterin stabilization occur in the reduced enzyme. These changes include different H-bonding interactions between surrounding amino acids and both pyranopterins, agreeing with previous reports on the role of the pyranopterin in fine-tuning the metal redox potential and enzymatic activity. Our combined results do not support a mechanism of Sec/Cys dissociation from the metal, and agree with a second coordination sphere mechanism that may involve hydride transfer. Nevertheless, more detailed studies with future variants and new crystal structures will be required to allow further detailed insights into the catalytic mechanism, and production of improved engineered forms of this important enzyme.

**MATERIALS AND METHODS**

**Expression of fdhAB.** The vectors used for homologous expression and StreptI tag insertion of DvFdhAB were both constructed using the Sequence and Ligation Independent Cloning—SLIC—method, as described previously. For the complementation vector plasmid pMO9075 was used as a source of the Desulfovibrio pBG1 replicon, the pUC origin of replication and the adaA gene, which confers resistance to spectinomycin — Sm8. In this strategy the full fdhA gene, including the signal peptide sequence, and the C-terminal strepII tag, was introduced with the 21 bp ribosomal-binding site on the pMO9075 plasmid. The primers were manufactured by Nzytech and are presented in Table S4. The fragments were treated separately with 0.5 U of T4 DNA polymerase (BioLabs Inc., New England), in the presence of 100 μg/mL of bovine serum albumin, for 60 min at room temperature, and the reaction was stopped by the addition of 1 mM DCTP. The treated fragments were mixed in a 1:1 molar ratio in T4 DNA ligase buffer (BioLabs Inc., New England) and incubated during 30 min at 37 °C. After a 10 min incubation on ice, 5 μL of the mixed fragments were transformed into 50 μL E. coli α-select Silver Efficiency (Bioline) and cells were plated on spectinomycin (100 μg/mL)-containing agar plates. The correct plasmid construct was screened by colony PCR and later confirmed by sequencing by GATC biotech, Germany.

For transformation, D. vulgaris and ΔfdhAB deletion strain were grown in MOY medium, with 30 mM sodium lactate/30 mM sodium sulfate (MOYLS) and 1.2 mM sodium thioglycolate. The constructed plasmids were then electroporated as previously described, the suicide vector was electroporated with 1500 V, 250 Ω, and 25 μF on the wild-type strain to induce double recombination; the complementation vector was integrated in the ΔfdhAB deletion strain by electroporation with 1250 V, 250 Ω, and 25 μF (Gene Pulse Xcell, Bio-Rad). Transformed cells were left to recover overnight at 37 °C in 1 mL MOYLS medium and then plated inside the gel box using MOYLS with 1.5% (w/v) agar, containing Geneticin G418 (400 μg/mL) and/or spectinomycin (100 μg/mL). Selected colonies were grown in liquid MOYLS and confirmed by PCR for fdhB gene. As a final confirmation, Streptagged DvFdhAB expression was verified by Western blot.

**Organism and Growth Conditions.** D. vulgaris Hildenborough (DSM 644) cells were grown in modified Postgate medium C containing 3.7 mM KH2PO4, 18.7 mM NH4Cl, 17.6 mM Na2SO4, 0.4 mM CaCl2·2H2O, 0.24 mM MgSO4·7H2O, 1 g/L yeast extract, 26 μM FeSO4·7H2O, 1 mM sodium citrate trisodium dihydroxide, 0.57 mM L-ascorbic acid, 0.88 mM sodium thiosulfate, 40 mM sodium formate, 10 mM sodium dithionate and 100 mM (12.1 g/L Tris base, supplemented with 1 μM of NiCl2·6H2O/Na2SeO3·5H2O and 0.1 μM of Na2O4·W2H2O). In the case of the ΔfdhAB complemented-strain, the concentration of Ni, Se, and W was increased to 10 μM. Moreover, to limit the concentration of Mo in the medium, the amount of yeast extract used was reduced to 0.4 g/L. Growth was performed at 37 °C in a 300 L reactor with N2 atmosphere. Cells were recovered by centrifugation and stored at −80 °C.

**Western Blot.** Cells were disrupted using BugBuster Protein Extraction Reagent (Novagen, Inc.). The soluble fraction was run on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane for 1 h at 350 V, mA and 4 °C in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). Membranes were blocked using PBS buffer with 3% BSA and 0.5% (v/v) Tween 20. The membrane was incubated with biotin blocking buffer (IBA Lifesciences, Germany) and then treated with Strept-Tactin antibody labeled with alkaline phosphatase (IBA Lifesciences, Germany) diluted 1:4000. After washing, immunodetection of bound antibodies was done, in the dark, by membrane treatment with ready-made solutions of nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyl phosphate—NBT-BCIP (Sigma), in 100 mM Tris-HCl pH 8.0, 100 mM NaCl, and 5 mM MgCl2. Reaction was stopped by washing the membrane with distilled water.

**Protein Purification.** For DvFdhAB purified, cells were resuspended, in 20 mM Tris-HCl pH 7.6 and 10 mM NaNO3 buffer, containing DNase, and disrupted in a French pressure cell in aerobic conditions. The crude extract was centrifuged at 40000 g for 20 min at 4 °C, and the supernatant was further clarified by ultracentrifugation at 100 000g for 2 h at 4 °C. All the purification steps were performed at 4 °C aerobically. The soluble fraction was then loaded on a Q-sepharose high performance column, equilibrated with 20 mM Tris-HCl pH 7.6 with 10% (v/v) glycerol and 10 mM NaNO3 (buffer A). Elution was carried out at a flow rate of 4 mL/min, with stepwise increments on NaCl concentration (50–200 mM steps, from 0 to 2400 mM). The buffer of the fractions containing FDH activity (eluted around 150 mM NaCl) was exchanged to 20 mM KPi (K2HPO4/KH2PO4 buffer) also with 10% (v/v) glycerol, and 10 mM NaNO3 (buffer B), using a 30-kDa cutoff ultracentrifugation unit (Amicon Ultra-15 30K NMWL, Millipore). Then, 15% (v/v) (NH4)2SO4 was added to the sample, which was further purified using a phenyl sepharose high performance column.

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equilibrated with buffer K with 15% (v/v) (NH₄)₂SO₄. Elution was performed by reducing the ionic strength in a stepwise decreasing gradient with a flow rate of 2 mL/min. Pure DvFdhAB was eluted between 10.5 and 12% (v/v) (NH₄)₂SO₄, and the purity of the eluted fractions was evaluated by 12% SDS-polyacrylamide gel stained with Coomassie Brilliant Blue R-250.

For recombinant DvFdhAB affinity purification, cells were resuspended in 100 mM Tris-HCl pH 8.0 with 10% (v/v) glycerol, 10 mM NaN₃, and 150 mM NaCl (buffer W). Cell disruption and soluble fraction clarification were performed as described above. The soluble fraction was then directly loaded on a Strep-tactin gravity flow (IBA Lifesciences, Germany) column equilibrated with buffer W. After five washing steps with buffer W, the recombinant protein was eluted with buffer W plus 2.5 mM d-desthiobiotin. The buffer of eluted samples was exchanged to buffer A and samples were stored, under nitrogen atmosphere, at 4 °C for immediate use, or −80 °C for longer periods. This column yields pure DvFdhAB as judged by 10% SDS-polyacrylamide gel.

Protein concentration was measured with the BCA Protein Assay Kit from Novagen, with bovine serum albumin as standard. The UV−visible absorption spectra of high purity oxidized sample was acquired on a UV-1800 Shimadzu spectrophotometer, and used to determine the molar extinction coefficient. The UV−visible spectrum was also recorded for DvFdhAB after reduction with 20 mM formate, for 30 min, and with 1 mM dithionite, inside the glovebox. Routine UV−visible absorption spectra were obtained with a Nanodrop ND2000C.

The molar extinction coefficient of the oxidized enzyme at 410 nm, was determined to be ε₄₁₀ nm = 43 446 M⁻¹ cm⁻¹.

**Thermal Shift Assay.** The melting temperature of DvFdhAB was determined using the Applied Biosystems Protein Thermal Shift Dye Kit: 1 to 2 mg/mL of pure enzyme, with 1 mM dithionite, inside the glovebox. Routine UV−visible absorption spectra were obtained with a Nanodrop ND2000C.

**Activity assays and kinetic analysis.** Activity assays were performed with small adaptations from previously reported protocols. FDH activity was determined using a UV-1800 Shimadzu spectrophotometer, inside a COY Anaerobic chamber, with an atmosphere of 2% H₂/98% N₂, at room temperature, with stirring. During assay optimization, different preincubations, their time, order of additions, and concentrations were tested. In the optimized method, the enzyme sample was preincubated with 50 mM DTT for 5 min prior to the reaction. Routinely, for formate oxidation measurements the reduction of 2 mM benzyl viologen was monitored at 555 nm (ε₅₅₅ nm (BV⁺) = 12 mM⁻¹ cm⁻¹), in 50 mM KPi pH 7.6 containing 1 mM DTT plus 20 mM sodium formate. One unit of oxidizing activity is defined as the amount of FDH capable of oxidizing 1 μmol of formate (reducing of 2 μmol of BV⁺) per min. For the reduction of CO₂, Zn-reduced methyl viologen was used as artificial electron donor. The oxidation of MV⁺ (0.1 mM) was followed at 578 nm (ε₅₇₈ nm (MV⁺) = 9.7 mM⁻¹ cm⁻¹), in 50 mM KPi pH 6.9 with 1 mM DTT plus 50 mM sodium bicarbonate, as a source of CO₂ (after the addition of bicarbonate the pH rises to approximately 7.1). One unit of reducing activity is defined as the amount of FDH capable of reducing 1 μmol of CO₂ (oxidizing 2 μmol MV⁺) per min. In assays with pure DvFdhAB a final concentration of 1.4 nM enzyme was used. The turnover numbers were calculated considering one catalytic active protomer αβ (138 kDa). To evaluate inhibition by nitrate and azide, measurements were performed in the presence of 100 mM of nitrate or 10 mM of sodium azide.

The influence of pH on FDH activity was assessed using a buffer mix containing 100 mM glycine, K₂HPO₄, citric acid, and Tris. The pH was adjusted using HCl or KOH. The activity of purified DvFdhAB was measured at substrate concentrations ranging from 0.5 μM to 10 mM sodium formate, at the optimum pH of 7.6. For the CO₂ reductase activity, concentrations of 75 μM to 50 mM sodium bicarbonate were employed at the optimum pH of 7.1 (see SI). In these assays, sodium bicarbonate was the last component to be added before starting the assay with the enzyme. Kinetic parameters were calculated by direct fitting of the Michaelis–Menten equation, using the least-squares method, using Graph Pad Prism 7.

**Iodoacetamide assays.** The effect of iodoacetamide (IAA) was measured on the formate-oxidation activity. We could not measure the IAA effect on CO₂ reduction because IAA oxidizes MV⁺ nonspecifically. As-isolated DvFdhAB was incubated for 30 min with 20 mM of IAA, alone or in the presence of 20 mM formate, or 20 mM nitrate, in 20 mM Tris HCl pH 7.6, 10% DTT and 1 mM dithionite. After that, the IAA-treated samples were incubated for 5 min with DTT prior to measurement of formate oxidation activities.

For mass spectrometry analysis, 10 μg of IAA-treated DvFdhAB were digested with trypsin overnight at 37 °C. The resulting peptides were analyzed by LC−MS, with data acquired in positive TOF-MS mode using X500B QTOF (ABSciex) with Turbo V ion source (Sciex) mass spectrometer connected to the ExionLC AD UPLC system. External calibration was performed using β-galactosidase digest (ABSciex). The raw MS and MS/MS data were analyzed using the Explorer software of SCIEX OS-Q, the BioPharmaView software 3.0 (Sciex) and the Protein Pilot for peptide identification. Data were obtained by the Mass Spectrometry Unit (UniMS), ITQB/iBET, Oeiras, Portugal.

**Crystallization, data collection, structure solution, and refinement.** For the crystallization of DvFdhAB, the commercial screens JBS 1, 2, 3, and 4 (Jena Biosciences) were used and 1 μL drops (1:1 protein:precipitant ratio) were dispensed using an Oryx8 crystallization robot (Douglas Instruments, Ltd.). The drops were set in 96 Well CrystalQuick X Plates (Greiner Bio-One) using the sitting-drop vapor diffusion method, and the plates were stored at 20 °C.

Thin needles appeared within 4 days on condition F11 (32% PEG 3350, 0.1 M Tris-HCl pH 8.5, 0.8 M LiCl) and were tested in situ using the home source (Bruker D8 Venture dual source X-ray generator equipped with a Photon 100 CMOS detector and the IXS stage). These crystals presented a protein diode pattern with very weak spots. In order to improve crystal quality, a scale up of the condition to 2 mL hanging drops was performed using X Plates (Greiner Bio-One) using the sitting-drop vapor vitamin C. The crystals grew as thin plates to a maximum size of 0.1 × 0.2 × 0.05 mm² in 7 days.

The crystallization of DvFdhAB in the formate-reduced form was performed in an anaerobic chamber under an argon atmosphere at <0.1 ppm of oxygen, and all the solutions used were previously degassed and stored in the anaerobic chamber. The protein was incubated with 10 mM of sodium formate, and the crystallization drops were prepared in the previously optimized condition (32% PEG 3350, 0.1 M Tris-HCl pH 8.5, 1 M LiCl). Crystals of the reduced form appeared after 1 week. 

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with the same shape of the oxidized form. The crystals were transferred into a cryoprotectant solution consisting of reservoir solution augmented with 20% (v/v) glycerol and then flash-cooled in liquid nitrogen.

X-ray diffraction data sets were collected at 100 k using a Pilatus 6M-F detector on ID29 and ID30 beamlines at the European Synchrotron Radiation Facility. The X-ray diffraction images were integrated with DLS,55 and data were scaled and merged using CCP4 program Aimless.60 The crystals of the oxidized form (DvFdhAB_red) belong to space group P2\_2\_2\_ with unit cell parameters a = 64.59 Å, b = 127.64 Å, and c = 148.21 Å and diffractions beyond 2.1 Å. The structure was solved by molecular replacement with Phaser57 using the structure of formate dehydrogenase from Desulfovibrio gigas to build the search model (PDB: 1H0H). The two protein sequences share around 64% identity, and the search model was prepared with the CHAINSAW58 tool from CCP4 software package, non-conserved residues were pruned down to the γ-atom. Crystals of the formate-reduced form (DvFdhAB_red) belong to the same space group with almost identical unit cell parameters a = 64.89 Å, b = 128.56 Å, and c = 149.63 Å and diffractions beyond 1.9 Å.

The structure was also solved by molecular replacement but using the oxidized form as search model. Model building and refinement were performed with Coot69 and phenix.refine.70 The refined models for oxidized and reduced forms (PDB entries 6SDR and 6SDV) contain 1177 and 1181 amino acid residues, respectively, and were assessed using Molprobity (Table S2). Data processing and refinement statistics are presented in Table S2.

Tunnel Calculation. The program CAVER 3.0.1 was used as a PyMOL plugin71 for calculating the tunnels in DvFdhAB, using manually adjusted settings. The initial starting point (S atom from W-SH), shell depth (2 Å), shell radius (3 Å), cluster threshold (3.5 Å), desired radius (5 Å), and maximum distance (3 Å) were maintained constant for all calculations. The maximum distance specifies the furthest point (from the initial point) to start a tunnel calculation. The bottleneck radius is the narrowest part of a given tunnel and corresponds to the minimum probe radius. The tunnel analysis provided a list of calculated tunnels that were ranked according to a specific cost function (e.g., for two tunnels with equal radii, the one with the shorter distances has a lower cost). The tunnel with the lowest cost was chosen as the primary tunnel.

Sequence Analyses. The amino acid sequence of D. vulgaris FdhA (UniProt- Q72E1) was used as query search against the UniProt reference proteomes via the Hidden Markov Model (HMM) profile implemented in the Phmmer web server.72 The search was performed using default parameters and 6063 sequences were found (June 2019), which include a range of different Mo/W oxidoreductases. These sequences were subjected to multistep clustering based on sequence identity at 95% (5848 clusters), 75% identity (2599 clusters), and 50% identity (608 clusters)), using the h-CD-HIT software73 present in the CD-HIT web suite.55

A Maximum Likelihood (ML) phylogenetic tree was built based on a multiple sequence alignment of the 608 sequences representing each cluster, performed with the MAFFT software74 using default options. Fourteen well-known FDH proteins separate into two different branches in the phylogenetic tree.55 One representing periplasmic NAD-independent FdhAs and containing 41 CD-HIT cluster representative sequences plus 10 known sequences (where D. vulgaris FdhA and E. coli FdhN and FdhO are included), and another representing cytoplasmic NAD-dependent FdhAs and containing 35 CD-HIT cluster representative sequences plus 4 known sequences (including E. coli FdhH). The multiple sequence alignment was edited using the Jalview software,76 where gaps were trimmed using D. vulgaris FdhA as reference. The consensus logo (Figure S14) was created with Jalview, based on the alignment of the 51 sequences from the branch of NAD-independent FdhAs.

EPR Spectroscopy. EPR spectra were recorded at X-band on a Bruker Elexsys 500 spectrometers equipped with an Oxford Instruments ESR900 helium gas flow cryostat. Formate-reduced crystals of DvFdhAB, prepared as described above, were transferred to an EPR tube inside the glovebox (Jacomex). A 57 solution sample of DvFdhAB (100 μL of 162 μM in Buffer A) was transferred to an EPR tube inside the glovebox and reduced by adding 20 μL of 60 mM sodium formate to the protein solution. EPR tubes were let to incubate for few minutes at room temperature, capped, transferred out of the glovebox, and frozen immediately in liquid nitrogen.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge on the ACS Publications Web site. The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.0c00086.

† Plasmid sequence maps; Western blot of DvFdhAB; expressing strains; SDS-PAGE gel of purified DvFdhAB; UV–visible spectrum of DvFdhAB; activity assays with different activation protocols; stability assays in different conditions; domains of DvFdhAB; electron density of DvFdhAB active site; EPR spectra of formate-reduced DvFdhAB in crystals and in solution; Gln890 environment in oxidized and reduced structures; formate and CO₂ tunnels; sequence conservation of residues lining the formate and hydrophobic tunnels; structural alignment of MGDs; calculations of dissolved CO₂ concentrations; crystallographic data processing and refinement statistics; reactivity of DvFdhAB cysteines with iodoacetamide; and primers (PDF)

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The authors declare no competing financial interest.

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