Inorganica Chimica Acta 455 (2017) 350–363

Contents lists available at ScienceDirect

Inorganica Chimica Acta

journal homepage: www.elsevier.com/locate/ica

Review article

Molybdenum and tungsten-containing formate dehydrogenases: Aiming to inspire a catalyst for carbon dioxide utilization

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ARTICLE INFO

Article history:
Received 18 May 2016
Received in revised form 1 July 2016
Accepted 2 July 2016
Available online 4 July 2016

Keywords:
Carbon dioxide reduction
Formate dehydrogenase
Molybdenum
Tungsten
Biocatalyst
Greener energy forms

ABSTRACT

The global energy demand and the present high dependence on fossil fuels have caused an unprecedented increase in the Earth's atmosphere carbon dioxide concentration. Its exponential and uncontrollable rise is responsible for large and unpredictable impacts on the world climate and for ocean acidification, thus, being a major concern for the ecosystems and human's daily life. On the other hand, the carbon dioxide abundance and low cost make it an interesting source for the production of chemical feedstocks and fuels. Yet, the thermodynamic and kinetic stability of the carbon dioxide molecule makes its utilization a laboratorial/industrially challenging task.

In this Review, we propose to use the molybdenum and tungsten-containing formate dehydrogenase (FDH) enzymes as a model to understand the mechanistic strategies and key chemical features needed to reduce carbon dioxide to formate. We will highlight the present knowledge about the structure of FDHs, with particular emphasis on active site features, reaction mechanism and ability to reduce carbon dioxide to formate. The information gathered aims to inspire the development of new efficient (bio)catalysts for the atmospheric carbon dioxide utilization, to produce energy and chemical feedstocks, while reducing an important environmental pollutant.

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Abbreviations: EPR, electron paramagnetic resonance spectroscopy; FDH, formate dehydrogenase; M, metal (refers to molybdenum and/or tungsten); Mo-FDH, molybdenum-containing formate dehydrogenase; W-FDH, tungsten-containing formate dehydrogenase; XAS, X-ray absorption spectroscopy.

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http://dx.doi.org/10.1016/j.ica.2016.07.010
0020-1693/

Isabel Moura received her Degree in Chemical Engineering from Technical University of Lisbon (Pt) in 1974. She received her Masters in Physical Inorganic Chemistry in 1977 and her Ph.D. from New University of Lisbon (UNL) in 1981 on the thesis entitled "Characterization of two types of iron sulfur centers in two proteins isolated from *Desulfovibrio gigas*". Since then she was an Assistant Professor until 1981 in the UNL. In 1986 she became an Associate Professor at UNL. She has done the habilitation in 1994 and became a Full Professor in 1997 at UNL. During her career she was a Visiting Professor in University of Geogia, Athens, USA. During the period 2000/2011 she was the Head of the Chemistry Department of FCT/UNL and the Director of the Associated Laboratory REQUIMTE for Sustainable Chemistry. Scientific interests: The main work aims on the study of Structure-Function of Metalloproteins. Application of biochemical and spectroscopic tools (NMR, EPR and Mössbauer). Proteins involved in relevant bacterial metabolic pathways – N and S Biocycles. Active sites include Cu, Co, hemes, iron-sulfur centers (rubredoxin type, [2Fe-4S], [3Fe-4S], [4Fe-4S]) as well as association of Fe-S centers with Mo, W, Ni, siroheme and flavins. New type of clusters and new metal associations is also one of the main topics of research. More than 385 publications, h-index of 56.

José J. G. Moura has a degree in Chemical Engineering and a Ph.D. in Chemistry, Professor of Chemistry at the Department of Chemistry, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa. The main field of research is Bioinorganic Chemistry and the role of Metals in Biology. More than 400 articles indexed in ISI Web of Knowledge and an h-index of 57. For extended periods Research Specialist at UMinnesota (US) and Adjunct Professor at UGeorgia, Athens (US). Past President of Chemistry Department and of Scientific Council at FCT-UNL, Portuguese Delegate to COST and INTAS, a member of Scientific Panel in the Calouste Gulbenkian Foundation and FCT/MCTES, NSF (US) Specialized Panel, and of several scientific editorial boards. In 2006, he was elected Member of Academia das Ciências de Lisboa and in 2010, elected President of the Society of Biological Inorganic Chemistry, for 2 years. Scientific key words: Bioinorganic, Biophysics, Biocatalysis, Energy Bioconversion (Hydrogen), Role of metals in Biology (heme and non-heme iron, molybdenum, tungsten, nickel, copper, vanadium and cobalt), Inorganic systems as models for biocatalysis, Spectroscopy (NMR, EPR and Mössbauer), (Bio) Electrochemistry, Protein-Protein interactions. Other interests: Director of FCT-UNL Campus FCT Library and Department of Documentation and Culture. Culture/Scientific Interfaces. Multidisciplinary curator activities (Art and Science).

Contents

1. The "carbon dioxide crisis

The global energy demand and the present high dependence on fossil fuels have caused the increase in the atmospheric carbon dioxide concentration for the highest values since records began [1]. Due to its significant green-house effect, carbon dioxide rise is responsible for large and unpredictable impacts on the world climate, besides being responsible for ocean warming and acidification (its major sink) [2,3]. While some authors defend that these alterations are no longer reversible, the carbon dioxide emissions must be greatly decelerate. Future energy sources should be carbon-neutral and based on solar, wind and geothermal energy and new methods to store, transport and use "on demand" the energy from these sources must be developed.

One solution to control the "carbon dioxide crisis" (alongside all other efforts to reduce emissions) would be the use of a renewable energy to scavenge the atmospheric carbon dioxide and convert it back into fuel [4]. Certainly, the carbon dioxide abundance makes it an attractive source for the production of fuels and other synthetic value-added chemicals and there is a huge interest in the development of strategies to efficiently scavenge and activate the atmospheric carbon dioxide [1,5–8]. One of the major challenges is the thermodynamic and kinetic stability of the carbon dioxide molecule that makes its laboratory/industrial activation a very difficult task. Nature, on the contrary, has found several different strategies to activate and use carbon dioxide [9–12], applying different chemical approaches, with specific enzymes, to cleave the C—O bond (reduction to carbon monoxide) and form C—C (*e.g.*, addition to ribulose 1,5-bisphosphate) and C—H bonds (reduction to formate) [8–14]. Understanding the chemical strategies already tested and proved by Nature (reaction mechanisms and key chemical features) would certainly contribute to the development of new efficient (bio)catalysts for the atmospheric carbon dioxide utilization [1,8,15–21].

This Review will be focused on the carbon dioxide reduction to formate catalyzed by formate dehydrogenase (FDH) enzymes. Formate is an interesting carbon dioxide product for several reasons: it is the first (and stable) intermediate in the reduction of carbon dioxide to methanol or methane; it is used as a chemical building block in industry; it is a viable energy source, easier to store and transport than dihydrogen (formate and dihydrogen are oxidized at similar potentials) and fuel cells that use formate are being developed. All these positive outcomes make worthwhile systematic investigations to develop more efficient (bio)catalysts

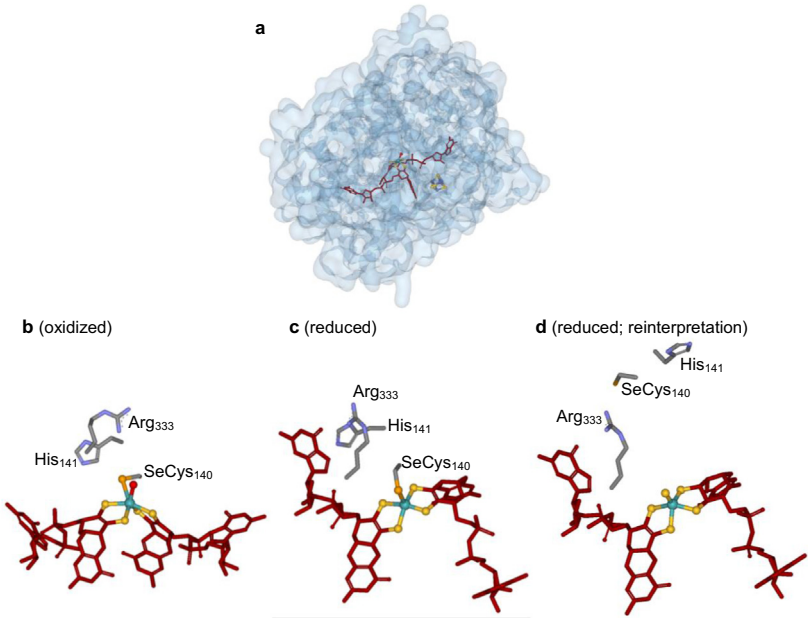

Fig. 1. *E. coli* formate dehydrogenase H. (a) Structural arrangement of the two redox centers, one molybdenum plus one [4Fe-4S] center, within the monomeric protein (α). (b) Active site of oxidized enzyme as described by Boyington et al. in 1997 [37]. (c) Active site of reduced enzyme as described by Boyington et al. in 1997 [37]. (d) Active site of reduced enzyme as described in the reinterpretation of Raaijmakers and Romão in 2006 [38].The structures shown are based on the PDB files 1FDO (a, b), 1AA6 (c) and 2IV2 (d). The pyranopterin cofactor is represented in dark red (each atom is indicated in Fig. 4a). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

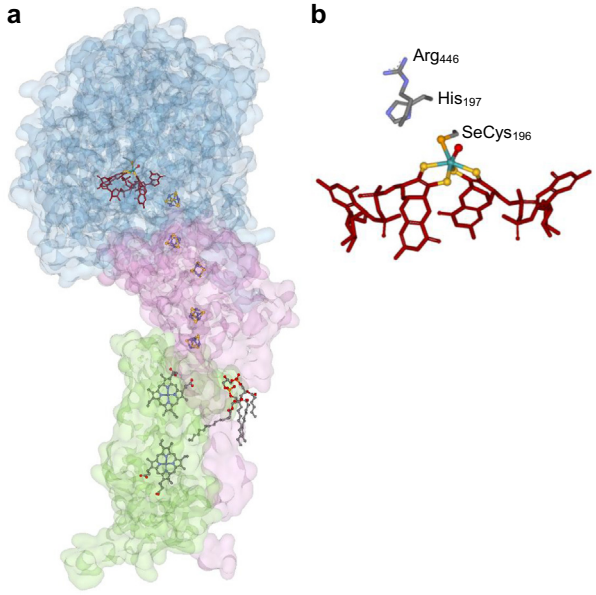

Fig. 2. *E. coli* formate dehydrogenase N. (a) Structural arrangement of the eight redox centers, one molybdenum, five [4Fe-4S] plus two *b*-type hemes, within the (αβγ) unit of the protein (α, blue; β, pink; γ, green). The cardiolipin molecule essential for the tight packing of the protein is also represented. (b) Active site of oxidized enzyme as described by Jormakka et al. in 2002 [40]. The structures shown are based on the PDB file 1KQF. The pyranopterin cofactor is represented in dark red (each atom is indicated in Fig. 4a). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

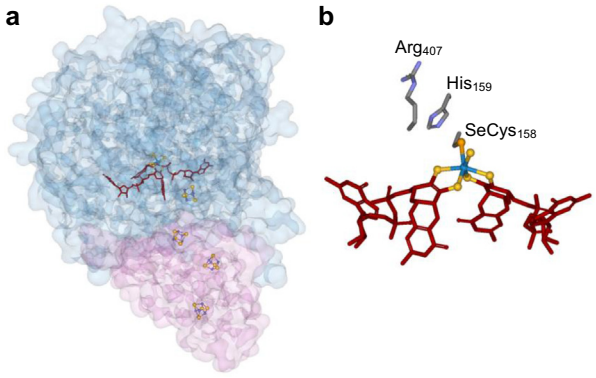

Fig. 3. *D. gigas* formate dehydrogenase. (a) Structural arrangement of the five redox centers, one molybdenum plus four [4Fe-4S] centers, within the dimeric protein (α, blue; β, pink). (b) Active site of oxidized enzyme as described by Raaijmakers et al. [52]. The structures shown are based on the PDB file 1H0H. The pyranopterin cofactor is represented in dark red (each atom is indicated in Fig. 4a). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to convert carbon dioxide into formate. We propose to use FDHs as an inspiration to decipher the key chemical features needed to convert carbon dioxide into formate. Herein, our present knowledge about the FDHs structure, active site features and mechanistic strategies will be reviewed and discussed (Section 2), followed by an account on the FDHs activity towards carbon dioxide reduction (Section 3).

2. Formate dehydrogenases

2.1. Families of formate dehydrogenases

FDHs catalyze the reversible two-electron oxidation of formate to carbon dioxide (Eq. (1)). The very low reduction potential value ($E^{o\prime}(CO_2/HCOO^-) = -0.43$ V [22]) drove prokaryotes to couple the formate oxidation to the reduction of several terminal electron acceptors, to derive energy. In addition, formate, the simplest carboxylate, is broadly used by both prokaryotes and eukaryotes in C1 metabolism.

Table 1
Summary of the features of some formate dehydrogenases.

Enzyme	Active site[a]	Subunit composition	Gene organiz.	Other	Reduce CO_2?[b]	Ref./Fig.
Desulfovibrio gigas FDH	W SeCys S	αβ α: W, [4Fe-4S] β: 3 [4Fe-4S]	*fdhA*, *fdhB*	• Periplasmatic	?	[50–52]Fig. 3
Syntrophobacter fumaroxidans FDH1	W SeCys S?	$(\alpha\beta\gamma)_2$ W, Fe	*fdsGBAC*	• Periplasmatic?	$k \approx 2.5 \times 10^3\ s^{-1}$	[138–141]
Moorella thermoacetica FDH (*Clostridium thermoaceticum*)	W SeCys S?	$(\alpha\beta)_2$ α: W, [4Fe-4S] β: 3 [4Fe-4S]	*fdhAB*	• Cytoplasmatic • $NADP^+$-dependent	Yes[c]	[146,161–164]
Clostridium carboxidivorans FDH	W SeCys S?	α W, [4Fe-4S]?	[d]	• Cytoplasmatic? • NAD^+-dependent	$k_{cat} = 0.08\ s^{-1}$	[147–149]
Methylobacterium extorquens FDH	W Cys S	αβ α: W, ⩾1 Fe/S β: [4Fe-4S], FMN	*fdh1A*, *fdh1B*	• Cytoplasmatic • NAD^+-dependent	Yes[c]	[165,166]
Escherichia coli FDH H	Mo SeCys S	α Mo, [4Fe-4S]	*fdhF*	• Cytoplasmatic (membrane-bound via its partners) • Partner of formate-hydrogen lyase complex	$k < 1\ s^{-1}$	[34–38,151,152] Fig. 1
E. coli FDH N	Mo SeCys S	$(\alpha\beta\gamma)_3$ α: Mo, [4Fe-4S] β: 4 [4Fe-4S] γ: 2 *b* hemes	*fdnGHI*	• Membrane-bound periplasm-faced • Partner in anaerobic nitrate-formate respiratory system	?	[39,40]Fig. 2
E. coli FDH O	MoSeCysS	$(\alpha\beta\gamma)_3$ α: Mo, [4Fe-4S] β: 4 [4Fe-4S] γ: 2 *b* hemes	*fdoGHI*, *fdhE*	• Membrane-bound periplasm-faced • Partner in nitrate-formate respiratory • System during aerobic to anaerobic transition	?	[41–43]
Desulfovibrio desulfuricans FDH	Mo SeCys S	αβγ α: Mo, [4Fe-4S] β: [4Fe-4S] γ: 4 *c* hemes	*fdhABC3*	• Periplasmatic	$k_{cat} = 46.6\ s^{-1}$	[53–55]
Desulfovibrio vulgaris Hildenborough $FDHABC_3$	Mo SeCys S?	αβγ α: Mo, [4Fe-4S] β: 3 [4Fe-4S] γ: 4 *c* hemes	*FdhABC3*	• Periplasmatic	$k \approx 3.4\ s^{-1}$	[44,45,47,49,153]
Acetobacterium woodii FDH	Mo SeCys S?	α Mo, [4Fe-4S]	*fdhF*	• Partner of hydrogen-dependent • Carbon dioxide reductase complex	$k_{cat} = 28\ s^{-1}$	[143,144]
Cupriavidus necator[e] FDH	Mo Cys S	αβγ α: Mo, 2 [2Fe-2S], 3 [4Fe-4S] β: [4Fe-4S], FMN γ: 2 [2Fe-2S]	*fdsGBACD*	• Cytoplasmatic • NAD^+-dependent	?	[103,115,167]
Rhodobacter capsulatus FDH	Mo Cys S	$(\alpha\beta\gamma)_2$ α: Mo, [2Fe-2S], 4 [4Fe-4S] β: [4Fe-4S], FMN γ: [2Fe-2S]	*fdsGBACD*	• Cytoplasmatic • NAD^+-dependent	$k_{cat} = 1.5\ s^{-1}$	[58,64,84]
Methylosinus trichosporium FDH	Mo Cys S?	$(\alpha\beta\gamma)_2$ Mo, ⩾1 [2Fe-2S], ⩾1 [4Fe-4S], FMN	*fdsGBACD*	• Cytoplasmatic • NAD^+-dependent	Yes[c]	[168,169]
Methanobacterium formicicum FDH	Mo Cys S	αβ Mo, FAD, several Fe/S, Zn	*fdhCAB*	• Cytoplasmatic • F_{420}-dependent	?	[114,170–175]

[a] Active site composition, besides the two pyranopterin cofactor molecules (Fig. 4).
[b] See Section 3 for details.
[c] No kinetic characterization available.
[d] Unknown gene organization.
[e] Previously known as *Alcaligenes eutrophus* and *Ralstonia eutropha*.

$$HCOO^- \rightleftharpoons CO_2 + 2e^- + H^+ \quad (1)$$

Hence, several FDHs evolved to take part in diverse biochemical pathways, in different subcellular locations and using various physiological redox partners (cytochromes, ferredoxins, NAD, coenzyme F_{420} or membrane quinols) (recently reviewed, *e.g.*, in [23–27]). With each pathway requiring a specific "enzymatic machinery" to accomplish the respective function, different FDHs have evolved: membrane-bound enzymes, that need specific "interfaces" to become "anchored" to the membrane and to interact with membrane-associated redox partners, and "free" cytoplasmatic and periplasmatic enzymes, that require other types of "interfaces" to recognize and exchange electrons/protons with the respective redox partners. As a result, FDHs are a group of heterogeneous proteins, harboring diverse redox-active centers or no redox centers at all, and displaying different subunit compositions and quaternary structures.

FDHs can be divided into two major classes, based on their metal content and the consequent chemical strategy followed by

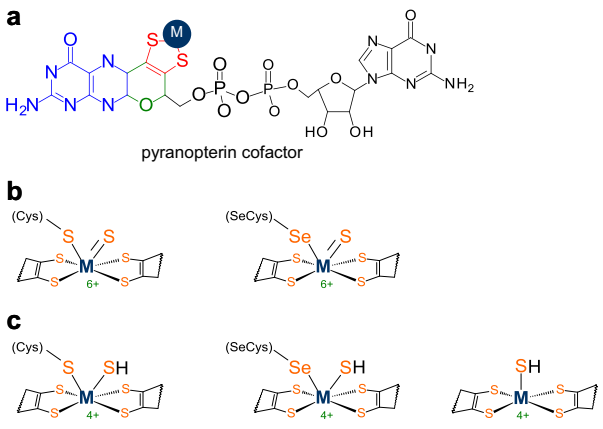

Fig. 4. The active site of formate dehydrogenases. (a) Structure of the pyranopterin cofactor. The pyranopterin cofactor molecule is formed by pyrano(green)-pterin (blue)-dithiolene(red)-methylphosphate(black) moieties; in FDHs, the cofactor is found esterified with a guanosine monophosphate (black). The dithiolene (—S—C=C—S—) group forms a five-membered ene-1,2-dithiolene chelate ring with the molybdenum or tungsten atom, here indicated as M (from metal). (b) Shows the structure that is consensually accepted for the oxidized state of the catalytic center of FDHs. (c) Depicts the structures suggested for the reduced state, with the cysteine or selenocysteine residue bound to the metal and with the residue dissociated as suggested by the reinterpretation by Raaijmakers and Romão [38]. In panels (b) and (c), for simplicity, only the dithiolene moiety of the pyranopterin cofactor is represented. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the active site to carry out the formate oxidation (Eq. (1)). One class comprises NAD-dependent enzymes that have no metal ions or other redox-active centers and belong to the *D*-specific dehydrogenases of 2-oxyacids family – *the class of metal-independent FDHs* [28–33]. These enzymes are widespread, being found in bacteria, yeasts, fungi and plants. The other class comprises only prokaryotic enzymes that hold different redox-active centers and whose active site harbors one molybdenum or one tungsten atom that mediates the formate oxidation – *the class of metal-dependent FDHs* [23–27]. The molybdenum-containing (Mo-FDH) and tungsten-containing (W-FDH) FDHs will be the focus of this Review[1].

2.2. Molybdenum and tungsten-containing formate dehydrogenases

Metal-dependent FDHs are structurally very diverse. *Escherichia coli*, *e.g.*, can express one "simple" monomeric cytoplasmatic enzyme that contains only the molybdenum center and one Fe/S center (the FDH H; Fig. 1a) [34–38] and two "complex" heteromeric ($(\alpha\beta\gamma)_3$) membrane-bound respiratory enzymes that harbor seven additional redox-active centers (Fe/S centers and hemes) besides the molybdenum center (the FDH N [39,40] (Fig. 2a) and FDH O [41–43]). Also the sulfate-reducing bacteria of the *Desulfovibrio* genus contain diverse Mo-FDHs and W-FDHs [44–49], such as the dimeric ($\alpha\beta$) periplasmatic W-FDH of *D. gigas*, with four Fe/S centers and one tungsten center (Fig. 3a) [50–52], or the more "complex" heteromeric ($\alpha\beta\gamma$) Mo-FDH of *D. desulfuricans*, that contains six redox-active centers (Fe/S centers and hemes) in addition to the molybdenum center [53–55]. The "enzymatic machineries" used by FDHs were recently reviewed [23–27] and the detailed information will not be duplicated here. Instead, Table 1 provides here an easily readable summary of the structural features and cellular roles of some representative FDHs.

Contrary to the diversity in structural organization and redox-active centers composition illustrated in Table 1, the active site of all presently known metal-dependent FDHs is well conserved [23–27]. These enzymes belong to the super-family of mononuclear molybdenum/tungsten-*bis* pyranopterin guanosine dinucleotide-containing enzymes (Mo/W-*bis* PGD) [23,25,27]. Their active site in its oxidized form harbors one molybdenum atom (in the case of Mo-FDHs) or one tungsten atom (in W-FDHs) coordinated by the *cis*-dithiolene (—S—C=C—S—) group of two pyranopterin cofactor molecules (Fig. 4a) and by one terminal sulfo group (Mo = S or W = S) plus one sulfur or selenium atom from a cysteine or selenocysteine residue, in a distorted trigonal prismatic geometry (Fig. 4b). Noteworthy, as far as is presently known, the presence of one selenocysteine or one cysteine residue in the metal coordination sphere is the only variable known in the active site of FDHs (besides the metal dichotomy). Although all known three-dimensional structures are from FDHs harboring a selenocysteine, Mo-FDHs and W-FDHs enzymes are known that do not have selenocysteine residues (Table 1), showing that there is no apparent relation between the metal and the amino acid chosen for the active site.

The first three-dimensional structure of a FDH, the one of *E. coli* FDH H determined by Boyington et al. in 1997 [37], was interpreted as indicating the presence of a labile hydroxyl group and not of a terminal sulfo group (Fig. 1b). Also Jormakka et al. in 2002 modeled the sixth ligand of the *E. coli* FDH N as a hydroxyl group (Fig. 2b) [40]. However, in the same year, Raaijmakers et al. [52] showed that the W-FDH of *D. gigas* contains instead a terminal sulfur atom in the metal coordination sphere (Fig. 3b). Following this assignment, Raaijmakers and Romão (2006) [38] reinterpreted the original electron density map of the *E. coli* FDH H (Fig. 1c) and proposed that the sixth ligand must be a sulfur atom (Fig. 1d). Besides the crystallographic data, also the recent identification of an *E. coli* sulfotransferase, which would catalyze the insertion of the sulfur atom [56,57], supports that all *E. coli* FDH would share the same active site structure, with a terminal sulfo group. A homologous sulfotransferase was also identified in *Rhodobacter capsulatus* and shown to be essential for the insertion of the sulfo ligand [58]. Hence, it is presently accepted that the oxidized form of the active site of Mo-FDHs and W-FDHs holds a terminal sulfo group (Fig. 4b).

The debate now lays in the reduced form of the FDHs active site. The molybdenum center of reduced *E. coli* FDH H was described by Boyington et al. [37] to lose the sixth ligand (assigned in 1997 as a hydroxyl group) and to acquire an approximate square pyramidal geometry, with the four sulfur atoms of the two pyranopterin molecules in the equatorial positions and the selenium atom of the coordinating selenocysteine residue in the axial position (Fig. 1c). However, in their reinterpretation of the crystallographic data, Raaijmakers and Romão (2006) [38] described that the polypeptide loop containing the selenocysteine was not properly traced in the original work and that the selenocysteine residue is, instead, found shifted away (12 Å) and dissociated from the molybdenum center (Fig. 1d). For that reason, those authors suggested that, while in the oxidized state the selenocysteine residue is coordinated to the molybdenum (Fig. 4b), the enzyme reduction triggers the dissociation of the residue from the molybdenum atom (Fig. 4c). The square pyramidal geometry of the molybdenum center was, thus, interpreted as arising from the presence of the terminal sulfo group in the axial position (Figs. 1d and 4c). This interpretation, uncoordinated selenocysteine (or cysteine) with an axial terminal sulfo group, also explains how an oxidized molybdenum (or tungsten) center, hexa-coordinated with a terminal sulfo group, can display a square pyramidal geometry after

[1] Of note, there are also NAD-dependent Mo-FDHs and W-FDHs (Table 1) and these enzymes should not be confused with the NAD-dependent metal-independent FDHs. Regardless of the use of NAD^+/NADH (co-substrate), these enzymes use a different "enzymatic machinery" to accomplish the formate oxidation, as will be discussed in Section 2.4.

reduction: a sulfo ligand can not be easily released (contrary to the initially proposed hydroxyl ligand that can be released after being protonated to yield a water molecule) and it is the selenocysteine/-cysteine that must be dissociated to give rise to the observed square pyramidal geometry.

However, presently, there are several lines of evidence suggesting that the FDHs active site selenocysteine/cysteine residue does not become dissociated from the molybdenum/tungsten atom after the enzyme reduction. All the presently available FDH crystal structures, with the exception of the work of Raaijmakers and Romão [38], were interpreted as showing the selenocysteine residue bound to the molybdenum [37] or tungsten [52] atom (Figs. 1–3). In addition, X-ray absorption spectroscopy (XAS) results obtained with FDHs from *E. coli* [59], *R. capsulatus* [58] and *D. desulfuricans* [60] suggest that both the oxidized and reduced molybdenum center hold the selenocysteine/cysteine bound to the molybdenum atom: the *E. coli* enzyme EXAFS data at both the Mo and Se K-edges were interpreted as indicating the presence of one Mo—Se bond of 2.62 Å, plus one Se—S bond of 2.19 Å (between the sulfo group and the selenocysteine selenium) [59]; a very similar selenium coordination was suggested for the *D. desulfuricans* FDH [60]; one Mo—S(Cys) bond of 2.63 Å was suggested for the *R. capsulatus* FDH [58]. Further experimental evidence came from electron paramagnetic resonance (EPR) spectroscopy that clearly showed that the selenocysteine/cysteine must remain bound to the Mo^{5+} center of formate-reduced enzyme [61,62]. When the EPR spectrum is obtained from ^{77}Se-enriched enzyme a very strong and anisotropic interaction with selenium is observed ($A_{1,2,3}(^{77}Se)$ = 13.2, 75, 240 MHz) [61]. This interaction and the observation of the expected ^{95,97}Mo hyperfine coupling confirms that the selenium atom of the selenocysteine is directly coordinated to the Mo^{5+} and further suggests that the unpaired electron is delocalized over the selenium (17–27%) and molybdenum atoms (73–83%) [61]. Also the hydrogen atoms of the β-methylene carbon of the selenocysteine residue are thought to be in the close proximity of the molybdenum atom, being responsible for an interaction with a not solvent-exchangeable proton (A_1 = 35.1 MHz) [54]. Photolysis assays additionally confirmed that the selenium/sulfur ligation is retained in the FDH Mo^{5+} center (the light beam did not affect the ^{77}Se interaction) [61]. The Mo^{5+} hexa-coordination (resulting from having the selenocysteine/cysteine residue bound to the molybdenum atom) was also supported by theoretical calculations on the signal-giving species of FDHs [63]. A different type of evidence was given by the very recent demonstration that the FDH activity is not affected by the iodoacetamide treatment (at pH 6 to 10) [64]. This alkylating agent reacts with "free" ionized selenocysteine and cysteine residues (carboxamidomethylation) and the absence of effect on FDH suggests that the active site selenocysteine/cysteine residue is bound to the molybdenum and not "free" to react with the iodoacetamide.

Hence, additional experimental work is needed to solve the structural ambiguities of the FDHs active site. It is possible that the crystallization/irradiation had induced some artifacts that are not relevant to the enzyme activity; but it is also possible that the species crystallographically characterized, being catalytically relevant, bear no relation to the species observed by XAS and EPR. Certainly, high resolution structures are needed to confirm the existence of the two alternating conformations of the selenocysteine/cysteine-containing polypeptide loop and to discuss the catalytic relevance of each conformation. Accordingly, the two possible coordination spheres of the reduced molybdenum/tungsten center are represented in Fig. 4b.

In addition to the molybdenum or tungsten center itself, the FDHs active site comprises also arginine and histidine residues that are strictly conserved in all known FDHs [37,38,40,52]. These residues are thought to be crucial to the catalytic cycle (as discussed below).

2.3. Mechanistic strategies for formate dehydrogenases

The molybdenum (Mo-FDH) or tungsten (W-FDH) center is the enzyme active site, where formate is oxidized and carbon dioxide is reduced (Eq. (1)). The other redox-active centers of each FDH (Table 1) are involved simply in the intramolecular electron transfer to/from the physiological acceptor/donor. The additional redox-active centers form a "wire" that facilitates the fast and effective electron transfer over a distance that can be very long, as is the case in *E. coli* FDH N, where the electrons are transferred against the membrane potential over the 90 Å that separate the molybdenum from the membrane menaquinone (from the α to the γ subunit; Table 1) [40]. Hence, the oxidation and reduction half-reactions are physically separated and, although the interconversion of formate and carbon dioxide only occurs in the molybdenum/tungsten center, the intramolecular electron transfer is, thus, an integral aspect of catalysis.

Formate oxidation is not an oxygen atom transfer reaction, as is characteristic of many molybdenum and tungsten-containing enzymes [23,25,27]: the product of formate oxidation is carbon dioxide and not hydrogencarbonate (Eq. (1) *versus* Eq. (2)) [65], as was clearly demonstrated by the formation of $^{13}C^{16}O_2$ gas during the oxidation of ^{13}C-labeled formate in ^{18}O-enriched water [61]. To accomplish this, FDH has to abstract one proton plus two electrons (Eq. (1)) or one hydride (Eq. (3)) from the formate molecule, in a mechanism that is believed to be identical in Mo-FDH and W-FDH.[2]

$$HCOO^- + H_2O \text{ —— does not occur ⟶ } HOCOO^- + 2e^- + 2H^+ \quad (2)$$

$$HCOO^- \rightleftharpoons CO_2 + 2e^- + H^+ \quad (1)$$

$$HCOO^- \rightleftharpoons CO_2 + H^- \quad (3)$$

One of the earlier FDH reaction mechanisms was supported by the first FDH three-dimensional structure of Boyington et al. (*E. coli* FDH H; 1997) [37] and it involved the release of the alleged hydroxyl group, to yield a penta-coordinated metal center to which formate is bound. Yet, the latter confirmation of the terminal sulfo group and the reinterpretation of the crystallographic data by Raaijmakers and Romão (2006) [38], indicating that the selenocysteine becomes dissociated from the molybdenum center in the formate-reduced *E. coli* FDH H, launch an innovative mechanistic concept involving [38,58,80–83]: (i) the selenocysteine/cysteine dissociation, (ii) the necessity of binding formate directly to the molybdenum atom or tungsten atom, M–OCO(H) (M stands for metal) and (iii) the proton abstraction by the selenium/sulfur atom from dissociated selenocysteine/cysteine residue.

Extended DFT calculations led to the comprehensive "sulfur shift mechanism" that, in general terms, can be divided into three main stages (Fig. 5) [81–83]. (i) In an initial activation stage, the hexa-coordinated molybdenum/tungsten center becomes penta-coordinated through the dissociation of the selenocysteine/cysteine in the presence of formate (Fig. 5(i) → (ii)). It is suggested

[2] For almost all known tungstoenzymes there is a homologous molybdoenzyme, either in the same or in different organisms [66]. This is the case of FDH, with several known W-FDHs and Mo-FDHs (Table 1). This general observation makes perfect sense when one takes into account the chemical similarities between tungsten and molybdenum and the fact that both metals are coordinated by the same pyranopterin cofactor molecule. Yet, if this reasoning is correct, *why are the vast majority of molybdoenzymes plus tungstoenzymes active only with molybdenum or with tungsten?* In fact, with a few exceptions (*D. alaskensis* [46,48] and *Desulfovibrio vulgaris* [49] FDHs, *R. capsulatus* dimethylsulfoxide reductase [67] and *E. coli* trimethylamine N-oxide reductase [68]), the substitution of molybdenum by tungsten [69–77], and vice versa [66,78–79], results in metal-free and tungsten/molybdenum-substituted enzymes that display no enzymatic activity. Clearly, the incorporation and role of each metal in active enzymes is far from being elucidated and these intriguing questions must wait for future research to be critically answered.

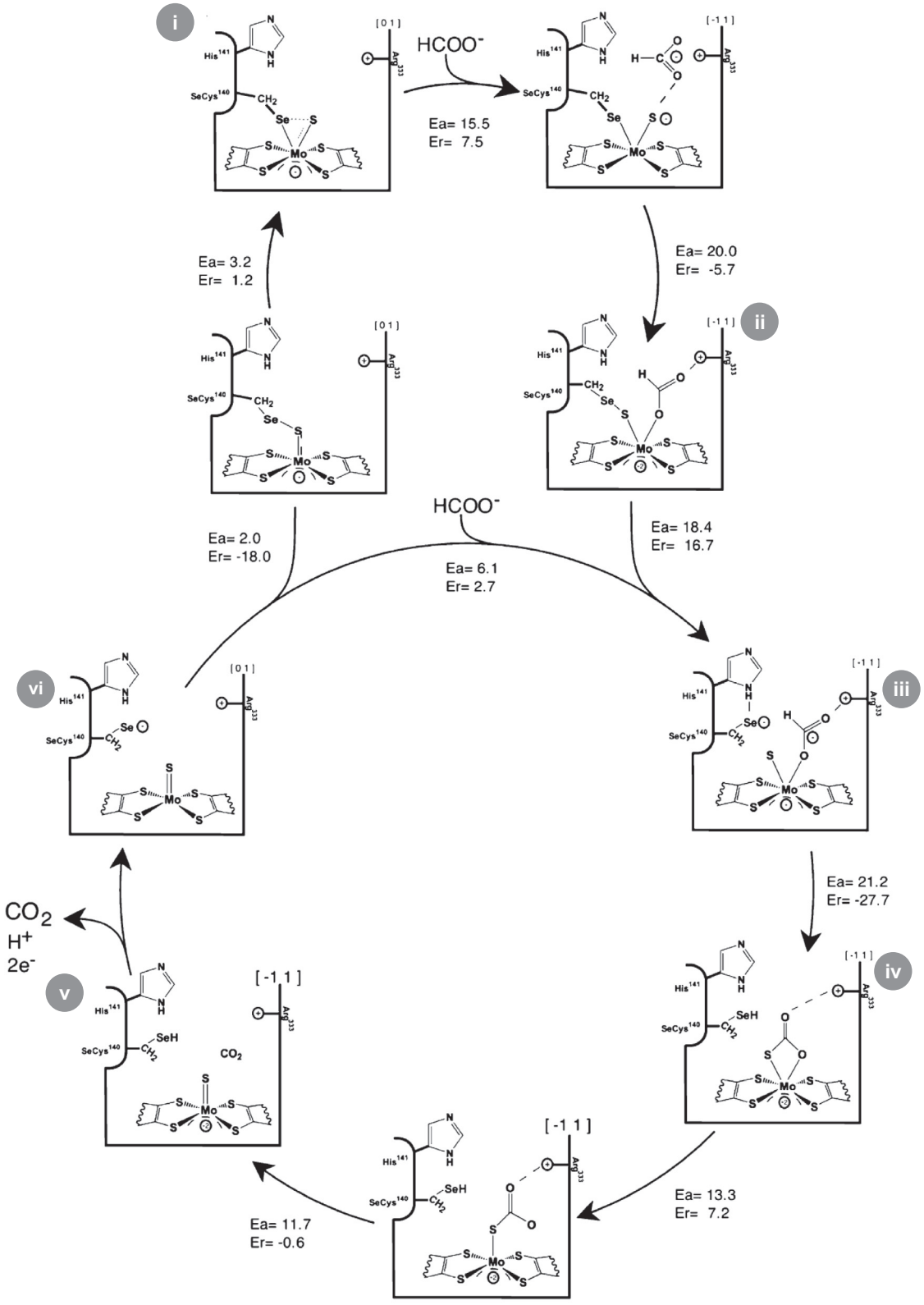

Fig. 5. The sulfur shift mechanism proposed for formate dehydrogenases. Reaction mechanism proposed for the FDH-catalyzed formate oxidation, as suggested by Mota et al. [81]. For simplicity, the mechanism is represented only for an enzyme containing an active site selenocysteine residue (it should be similar for a cysteine-containing FDH). See text for details. The E_a and E_r values are expressed in kcal/mol. Adapted with permission from [81].

that when formate approaches the molybdenum/tungsten center, with its oxygen moving toward the sulfo group (M = S), it pushes the sulfur to the position originally occupied by the selenocysteine/cysteine residue, to yield a (Cys)Se/S—S(M) bond. In this process, the metal is formally reduced to M^{4+} (intramolecular reduction at the *expenses* of making a persulfide Se/S—S bond) and a vacant coordination position is created in the metal coordination sphere, to which formate binds to form a reduced M^{4+}–OCO(H) complex (Fig. 5(ii)). (ii) In the catalytic part, the (Cys)Se/S—S(M) bond is cleaved and the uncoordinated selenocysteine/cysteine residue (—(Cys)Se^-, or —(Cys)S^-), stabilized by a hydrogen bond with the conserved histidine, abstracts the formate Cα proton (to yield —(Cys)SeH, or —(Cys)SH) (Fig. 5(iii) → (iv)). A carbon dioxide moiety is formed attached to the metal (M–S–CO_2), which is eventually released and a penta-coordinated reduced center is formed, with the sulfo group as the fifth axial ligand, M^{4+}=S (Fig. 5(v)), in accordance with the X-ray data interpretation of Raaijmakers and Romão [38]. (iii) In a final stage, the catalytic cycle is closed with the oxidation of M^{4+} to M^{6+}, via intramolecular electron transfer to other(s) redox center(s), and the deprotonation of the selenocysteine/cysteine residue (Fig. 5(v) → (vi)). The molybdenum/tungsten center can, then, bind a new formate molecule and start a new catalytic cycle; in the absence of formate, the selenocysteine/cysteine-containing loop is reoriented, the (Cys)Se/S—S(M) bond reformed and the enzyme returns to the inactive hexa-coordinated form (Fig. 5(i)).

Accordingly to the sulfur shift mechanism, the conserved positively charged arginine plays a key role in driving the formate anion into the active site and its subsequent binding to the molybdenum/tungsten in the correct position; it would also play a role in product release. The conserved histidine plays a key role in stabilizing (through a hydrogen bond) the uncoordinated selenocysteine/cysteine; accordingly to this mechanism, this is essential to facilitate the formate Cα proton abstraction by the selenocysteine/cysteine. The role of direct proton acceptor given to the selenocysteine/cysteine residue is in agreement with the pH values for maximal activity of selenocysteine and cysteine-containing FDHs: a cysteine with a side chain pK_a value of ≈8.2, allows the existence of a (Cys)S^- anion at the "optimum pH" of 9.0 of *R. capsulatus* FDH [84]; the lower pK_a value of a selenocysteine side chain, of ≈5.2, is more "wide-ranging" and suitable for the "optimum pH" of 7.0–7.5 of *E. coli* FDH H[3]. The terminal sulfo group, M = S, however, does not play any relevant role in the sulfur shift mechanism, being suggested to only contribute to "anchor" transiently the carbon dioxide moiety to the molybdenum/tungsten (via a M–S–CO_2 complex; Fig. 5).

More recently (2012), a different mechanistic strategy was proposed by Tiberti et al. [86]. Based on theoretical calculations, these authors suggested that the formate oxidation occurs through the hydride transfer (Eq. (3)) from the formate molecule to the molybdenum atom itself, resulting in the formation of a Mo–H intermediate. Subsequently, the proton is transferred from the molybdenum to the selenocysteine/cysteine, which acts as the final proton acceptor. Thus, contrary to the above discussed mechanistic proposals, where the formate Cα hydrogen atom is transferred, in the form of a proton (Eq. (1)), directly to the selenocysteine/cysteine, *here* the molybdenum center has the unprecedented role of mediating the hydrogen transfer, in the form of a hydride (Eq. (3)), from the formate to the selenocysteine/cysteine residue. While explaining the general features of FDH reaction, this mechanistic proposal faces major criticisms, because it goes against the well documented and long known chemistry of molybdenum and tungsten [25,27,87–102]. Its chemistry is dominated by the formation of oxides and sulfides, with no precedent of redox-active Mo–H complexes.

2.4. A new look at the formate dehydrogenase catalysis

Presently (2015–2016), new experimental results and a careful rethinking of the *old* data are seeding novel ideas that point towards a different chemical strategy for the FDH reaction. One intriguing feature of the FDH catalysis is the use of molybdenum/tungsten in a reaction that does not involve oxygen or sulfur atom transfer, as is characteristic of almost all known molybdenum and tungsten-containing enzymes [23,25,27]. Typically, the molybdoenzymes and tungstoenzymes catalyze the transfer of an oxygen atom from water to product – *oxygen atom insertion* – or from substrate to water – *oxygen atom abstraction* – in what was coined by Holm and others in the 80 s as the "oxo transfer hypothesis" [25,27,87–102]. In these reactions, the molybdenum/tungsten atom cycles between M^{6+} and M^{4+} and, most important, the oxidized cores act as direct oxygen atom donors to the substrate, while the reduced cores act as oxygen atom acceptors from the substrate (Eqs. (4a) and (4b), respectively). This well documented chemistry raises two very puzzling questions: since the FDH-catalyzed reaction does not involve the transfer of an oxygen atom (Eq. (2)) [61,65], *do formate or carbon dioxide bind to the molybdenum/tungsten atom through the "expected" M^{6+}–OCO(H) or M^{4+}–OCO complexes?* And, if formate or carbon dioxide binds directly to the molybdenum/tungsten atom, *why the FDH molybdenum/tungsten center does not insert or abstract an oxygen atom into formate or from carbon dioxide* (Eq. (4))?

$$M^{6+}\text{-}O + X \longrightarrow M\text{-}O\text{-}X \longrightarrow M^{4+} + X\text{-}O \quad (4a)$$

$$M^{4+} + X\text{-}O \longrightarrow M\text{-}O\text{-}X \longrightarrow M^{6+}\text{-}O + X \quad (4b)$$

The relevance of these purely theoretical questions is emphasized by the fact that, as discussed above, there is no experimental consensus regarding the dissociation of the selenocysteine/cysteine residue from the molybdenum/tungsten center (if formate binds to the metal, then, the dissociation is necessary to create a vacant position in the metal coordination sphere). Furthermore, there is also no clear-cut evidence that formate/carbon dioxide binds directly to the FDH molybdenum/tungsten atom: there is no crystal structure showing the formate molecule in the active site[4] and the EPR signals obtained with different FDHs reduced with formate do not arise from FDH-formate complexes [54,61,103]. There is no evidence from other spectroscopic methods showing the binding of formate to molybdenum, except for the recent XAS at the Mo K-edge study of Schrapers et al. [58]. These authors suggested that, in the presence of formate, the cysteine ligation is replaced by a long Mo—O bond of 2.2 Å, which they interpreted as arising from the Mo–OCO(H) complex. However, the observation of unidentified Mo—X bonds and of further Mo—O bonds in formate-reacted FDH might question that assignment.

Hence, in the absence of more definitive experimental work, it can only be concluded that the direct formate binding to the molybdenum/tungsten might not occur, be necessary, or even desirable (Eqs. (1) or (3) *versus* Eqs. (2) and (4)). So, *how can the interconversion between formate and carbon dioxide be accomplished?*

A different chemical strategy for the FDH catalysis becomes evident when the focus is shifted from the selenocysteine/cysteine and turned into the terminal sulfo group of the active site. This metal sulfo group is well documented and consensually recognized

[3] In fact, the pK_a values of both amino acids have been evoked to explain why the replacement of the selenocysteine by a cysteine in *E. coli* FDH-H resulted in a ≈300 fold decreased activity at pH 7.5 [85].

[4] Boyington et al. [37] described the structure of FDH treated with the inhibitor nitrite, which shows the selenocysteine bound to the molybdenum atom and the nitrite molecule with one of its oxygen atoms at 2.58 Å from the molybdenum.

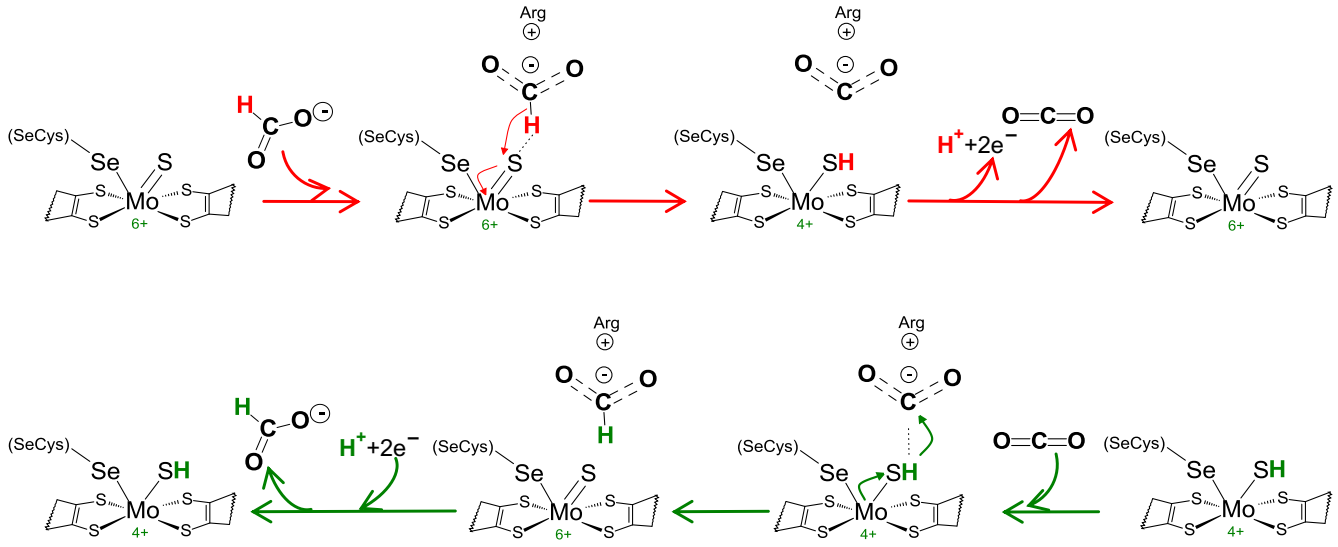

Fig. 6. The hydride transfer mechanism proposed for formate dehydrogenases. Reaction mechanism proposed for the FDH-catalyzed formate oxidation (red arrows) and carbon dioxide reduction (green arrows) [55,103]. The two reactions were represented separately to be easer to follow the individual steps in each case, but the red and green arrows represent one unique reversible reaction, that can be driven in both directions, depending on the prevalent molybdenum oxidation/reduction state. For simplicity, the mechanism is represented only for an enzyme containing an active site selenocysteine residue (it should be similar for a cysteine-containing FDH). See text for details. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

as a hydride acceptor/donor. Since the 70s, this group is established as the hydride acceptor in the oxidized molybdenum center (Mo^{6+}═S) of xanthine oxidase and aldehyde oxidase [25,27,104–108] and of other enzymes of the xanthine oxidase family [25,27], as well as the hydride donor in the reduced center (Mo^{4+}—SH) of hydroxybenzoyl-CoA reductase [109–110]. The highly covalent terminal sulfur atom of the metal sulfo group, with an available S π-bond, is well suited to accept a hydride and its pK_a value supports this "twin" behavior: the pK_a values of the coordinated ligands change dramatically with the oxidation state of the metal and determine that the higher oxidation states should hold deprotonated ligands (Mo^{6+}═S), while the lower oxidation states should hold protonated ligands (Mo^{4+}—SH) [111–113]. This terminal sulfur atom can be removed by treatment with cyanide, to yield a catalytically inactive desulfo-form that harbors a Mo = O instead of the native Mo = S group, which demonstrates the crucial role that the sulfo group plays in the catalysis of those enzymes [25,27,104,106,107].

The FDHs active site harbors the same terminal sulfo group (at least those FDHs whose crystal structure is known) and the FDHs are equally (and completely) inactivated by cyanide (*Methanobacterium formicicum* [114], *Cupriavidus necator* [115], *E. coli* [56] or *D. desulfuricans* [55] enzymes), most likely by a parallel abstraction of the terminal sulfur atom. Therefore, it is reasonable and predictable that the sulfo group plays a similar role in FDH, acting as a hydride acceptor in catalysis.

This claim is further supported by EPR studies showing that the formate Cα hydrogen atom is transferred to an acceptor group located within magnetic contact to the molybdenum atom of FDHs from different sources (*E. coli* [61], *D. desulfuricans* [54] or *Cupriavidus necator* [103]). The observation of a strongly coupled, solvent-exchangeable and substrate-derived proton, with a hyperfine constant of 20–30 MHz, is consistent with the hydrogen atom being transferred to a ligand in the first coordination sphere of the molybdenum atom [62]. Similar hyperfine constant values were determined in xanthine oxidase, where the strong coupled proton is originated from the xanthine C8 hydrogen atom (the position that is hydroxylated by that enzyme) [116–119]. The magnitude of the FDH hyperfine interaction could not arise from the transfer of the formate Cα hydrogen atom to a selenocysteine/cysteine residue that has been pushed away (9–12 Å [38,81]) from the molybdenum/tungsten atom. Photolysis assays (mentioned above) additionally support that the selenocysteine/cysteine is not the hydrogen acceptor: while the light beam did not affect the ^{77}Se interaction, it induced the photolysis of the solvent-exchangeable proton [61], indicating that the selenocysteine/cysteine ligation is retained in the FDH Mo^{5+} center, but it is not that residue that binds the strongly coupled proton. Therefore, for the catalytic cycle (that would run between M^{6+} and M^{4+}), it can be inferred that (i) if the selenocysteine/cysteine is dissociated from the molybdenum/tungsten during catalysis, it is not the hydrogen atom acceptor; (ii) if the selenocysteine/cysteine remains bound during catalysis, it would not be able to accept the hydrogen atom[5]. Hence, the only metal ligand that can accept the formate Cα hydrogen is the sulfo group and these EPR results are now being interpreted [55,103] as clearly supporting a hydride transfer from the formate molecule to the molybdenum/tungsten sulfo group and, then, to the solvent.

According to the above reasoning, the formate oxidation should occur through hydride transfer (Eq. (3)) and not via one proton plus two electrons transfer (Eq. (1)), as previously suggested. In fact, the abstraction of one hydride from the formate molecule should be more facile than the abstraction of one proton, because, in the first case, the direct reaction product is the final, stable carbon dioxide molecule, while, in the second case, there is a carbanion, $(CO_2)^{2-}$, whose formation is dependent on a very high pK_a value (pK_{a1} = 3.75 and pK_{a2} > 14). Further evidence that formate is intrinsically a good hydride donor comes from the "simple" (without redox-active centers) metal-independent FDHs. The chemistry behind the catalysis by these NAD-dependent enzymes is surprisingly simple [28–33]: the enzyme binds formate and NAD^+ in close proximity of each other (1.4 Å distance between H-(formate) and C4-(pyridine ring)) and makes NAD^+ acquire a bipolar conformation, which increases the electrophilicity and, thus, facilitates the hydride transfer. The reaction, then, proceeds by straightforward hydride transfer from formate to NAD^+. The parallel evidence that carbon

[5] It is not chemically reasonable to have a selenium/sulfur atom with three bonds and a positive charge, one bond to the amino acid side chain, plus one bond to the metal and another bond to the hydrogen. Theoretical calculations of the activation energy for the C—H bond cleavage also showed that the proton abstraction by a bound selenocysteine is highly unfavorable (36 kcal/mol) [80].

dioxide can accept a hydride comes from several synthetic transition metal–hydride complexes that mimic the FDH catalysis [120–124].

Following these novel ideas, it was suggested, originally for formate oxidation (*Ralstonia eutropha*) [103] and shortly after also for carbon dioxide reduction (*D. desulfuricans*) [55], that the FDH catalysis proceeds through hydride transfer, with the oxidized and reduced active site sulfo group, M^{6+}=S and M^{4+}—SH, acting as the direct hydride acceptor and donor, respectively. The formate oxidation (Fig. 6, red arrows) is initiated with the formate binding to the oxidized active site, but not directly to the molybdenum/tungsten atom. Following the example provided by the metal-independent FDH, whose formate binding site harbors arginine and asparagine residues [28–33], it is suggested that the conserved arginine, and possibly histidine, of Mo/W-FDHs are essential to drive the Cα hydrogen of formate towards the sulfo ligand by establishing hydrogen bond(s) with its oxygen atom(s). Also azide (N_3^-, isoelectronic with carbon dioxide) is suggested to bind (tightly) to the same site and not directly to the molybdenum/tungsten atom (as had been previously suggested for the *D. desulfuricans* FDH inhibition by azide [54,63]). The binding of azide and formate to the same site, and not to the molybdenum/tungsten atom itself, explains why azide is a powerful inhibitor of both metal-independent [30] and metal-dependent FDHs [54,61]. A similar reasoning applies to the inhibitor nitrite (isoelectronic with formate). Formate oxidation, then, proceeds by a straightforward hydride transfer from formate to the sulfo group of the oxidized molybdenum/tungsten center, M^{6+}=S, leading to the formation of M^{4+}—SH and carbon dioxide (Fig. 6, red arrows). The oxidation of M^{4+} to M^{6+}, via intramolecular electron transfer to other(s) redox center(s), and the release of carbon dioxide close the catalytic cycle. The now oxidized M^{6+} favors the sulfo group deprotonation (because of its pK_a value [111–113]) and the initial oxidized molybdenum/tungsten center, M^{6+}=S, is regenerated. Under non-steady-state catalytic conditions (as the ones created in EPR experiments) the molybdenum/tungsten one-electron oxidation should be favored ($M^{4+} \rightarrow M^{5+}$), leading to the formation of the EPR detectable Mo^{5+}—SH species.

The carbon dioxide reduction is suggested to follow the reverse reaction mechanism (Fig. 6, green arrows). First, carbon dioxide binds to the reduced active site, but not directly to the molybdenum/tungsten atom, at the same site as formate (and azide), with the conserved arginine (and possibly histidine) anchoring its oxygen atom(s) through hydrogen bond(s) and orienting its carbon atom towards the protonated sulfo ligand. Based on the inhibition and Michaelis constants for the *D. desulfuricans* FDH, the "binding strength" is suggested to follow the order carbon dioxide ($K_m \approx 15$ μM [55]) > azide ($K_i \approx 30$ μM [54]) > formate ($K_m \approx 60$ μM [55]). Then, the reaction proceeds through straightforward hydride transfer from the protonated sulfo group of the reduced molybdenum/tungsten center, M^{4+}—SH (as its pK_a suggests [111–113]), to the carbon dioxide carbon, whose LUMO have predominant C-*p* orbital character (Fig. 6, green arrows). This yields a formate moiety and M^{6+}=S. The subsequent reduction of M^{6+} to M^{4+}, via intramolecular electron transfer from other(s) redox center(s), and formate release closes the catalytic cycle. The now reduced M^{4+} favors the sulfo group protonation (pK_a value [111–113]) and the initial reduced molybdenum/tungsten center, M^{4+}—SH, is regenerated.

Hence, the novel hydride transfer mechanism points toward an "universal" chemical strategy for the FDH catalysis: formate binding in a close proximity to an oxidized, electrophilic, hydride acceptor, which in metal-independent enzymes is a NAD^+ molecule and in metal-dependent enzymes is the M^{6+}=S group. The reverse reaction is driven through the carbon dioxide binding in a close proximity of a reduced hydride donor, a NADH molecule or the M^{4+}—SH group. Overall, it is anticipated that the equilibrium between formate oxidation *versus* carbon dioxide reduction is determined by the ratio of NAD^+ *versus* NADH and of M^{6+} *versus* M^{4+}, which, in turn, determines also the protonation state of the metal sulfo group in a concerted and straightforward way. Regarding the reducing equivalents obtained from formate oxidation, they are transferred to the oxidizing substrate (or the reverse for the reducing equivalents consumed in carbon dioxide reduction, that are obtained from the reducing substrate). In the metal-independent FDHs, they are directly transferred to the NAD^+ molecule; in the metal-dependent FDHs, they are first transferred to the other (s) redox-active center(s) of the enzyme and, finally, to the oxidizing substrate (which, in the case of NAD-dependent Mo-FDHs and W-FDHs (Table 1), is also a NAD^+ molecule (see note 1)).

One limitation of the hydride transfer mechanism is that it does not assign any relevant role to the active site selenocysteine/cysteine residue. In fact, the hydride transfer to/from the sulfo group does not seem to require that the selenocysteine/cysteine remains bound to the molybdenum/tungsten atom (that is, the catalytic cycle, as represented in Fig. 6, could operate as well in a penta-coordinated molybdenum/tungsten center, with a dissociated selenocysteine/cysteine). If this is the case, *why do organisms spend energy to incorporate this residue? Why does either a selenocysteine or a cysteine fulfill the "job"* (Table 1)? And, if a cysteine can do the "job", *why do some organisms incorporate the biologically "more expensive" selenocysteine? Why not a different amino acid residue?*

It can be argued that a permanent hexa-coordination sphere is needed to hinder the oxygen (or sulfur) atom transfer activity that is characteristic of the molybdenum/tungsten-containing enzymes and, thus, allow the FDH to act exclusively as a hydrogen atom transfer enzyme. Yet, it seems plausible that the presence of a selenocysteine selenium (more covalent) or of a cysteine sulfur (less covalent) should alter the reaction energetic pattern. *Are the cysteine-containing enzymes compensating the absence of a selenium by introducing small changes in the active site?* (as described for other proteins where either Se and S can be found, *e.g.* [125]; recall that all three-dimensional structures known are from selenocysteine-containing FDHs).

In this respect, it is interesting to briefly consider the periplasmatic nitrate reductases from *D. desulfuricans* and *Cupriavidus necator* [126–128]. The molybdenum center of these enzymes shares the same hexa-coordination depicted in Fig. 4B (with a cysteine residue bound to the molybdenum atom). In these enzymes, the creation of a vacant coordination position is essential for the direct nitrate binding through one of its oxygen atoms, Mo^{4+}—ONO_2. It is that oxygen atom that will be, subsequently, abstracted by the enzyme active site, to yield nitrite and a Mo^{6+}=O center, in a "classic" molybdenum-dependent oxygen atom transfer reaction [25,27]. To create the vacant position, it was originally suggested the sulfur shift mechanism [128], through which the Mo^{6+}=S(—S(Cys)) center is converted into Mo^{4+}—ONO_2(—S—S(Cys)), in the presence of nitrate. Since the FDH catalysis does not involve the transfer of an oxygen atom (Eq. (2)), it is plausible that, contrary to those nitrate reductases, the FDH active site/substrate binding pocket evolved to disfavor the selenocysteine/cysteine residue dissociation and, thus, hold a permanently hexa-coordinated molybdenum center.

In addition, it can be suggested that a sulfur/selenium rich coordination of FDHs is needed to increase the covalency of the M—S bond of the sulfo group and/or to modulate the metal reduction potential, which, in turn, would facilitate the formate C—H bond heterolysis and hydride transfer. In the absence of further experimental evidences, both scenarios – *dissociated and bound selenocysteine/cysteine* – seem to be possible and this is an aspect that will remain open for now. Certainly, future research will shed light in these aspects of the FDH reaction, allowing a critic evaluation of this and previous mechanistic proposals.

3. Formate dehydrogenase-catalyzed carbon dioxide reduction

The interconversion between formate and carbon dioxide is feasible, as long as an oxidant or a reducer, with an appropriate reduction potential relative to the redox pair $CO_2/HCOO^-$, is present to drive the reaction in the direction of formate oxidation or carbon dioxide reduction, respectively ($E^{o\prime}$ (pH 7, formate 1molal activity, CO_2 (g) 1 atm) = −0.43 V [22]). Nevertheless, the highly negative value of the reduction potential of the $CO_2/HCOO^-$ pair anticipates that the carbon dioxide reduction would be thermodynamically difficult to accomplish. In fact, carbon dioxide is a thermodynamically and kinetically stable molecule, difficult to laboratorial/industrially activate and reduce.

The FDH-catalyzed formate oxidation would also be a reversible reaction if the FDH molybdenum/tungsten center can be poised at the appropriate reduction potential, to generate M^{6+} or M^{4+}, in order to make the reaction thermodynamically feasible in both directions *in vitro*[6] (Eq. (5)).

$$HCOO^- + FDH(M^{6+}) \rightarrow CO_2 + FDH(M^{4+}) + H^+ \quad (5a)$$

$$CO_2 + FDH(M^{4+}) + H^+ \rightarrow HCOO^- + FDH(M^{6+}) \quad (5b)$$

Although obvious, this is an aspect that is not always taken into consideration, particularly when the objective is to drive the reaction toward the carbon dioxide reduction *in vitro*. To accomplish this reduction, an efficient "electron source" is needed for the molybdenum/tungsten reduction in the presence of the other(s) redox-active center(s) of the enzyme: if the reduction potential of one (or more) of the redox centers is (are) relatively high, it could be difficult to "push" the electrons into the active site (the center with the higher reduction potential could stay reduced, "blocking" the electron transfer to the other(s) center(s) with lower reduction potentials). In this scenario, it is possible that the reaction is easier at an electrode surface (that can provide "endless" electrons with the suitable driving force) than in solution using an artificial reducing compound (that can be inefficient and slow comparatively to the enzyme). Yet, with the electrochemical methodologies, the unknown and the challenge is always the "enzyme-electrode communication".

The same reasoning applies to the *in vitro* formate oxidation reaction, when it is difficult to maintain the active site oxidized (to "pull" the electrons out of the active site). Besides these possible thermodynamic constrains also the kinetic feasibility of both formate oxidation and carbon dioxide reduction has to be taken into account to evaluate the reversibility of the FDH reaction and the effective carbon dioxide reduction (practical rate constant for oxidation (k^{HCCO-}) *versus* practical rate constant for reduction (k^{CO2}); Eq. (6)).

$$HCOO^- + A_{ox} \xrightarrow{k^{HCCO-}} CO_2 + A_{red} + H^+ \quad (6a)$$

$$CO_2 + A_{red} + H^+ \xrightarrow{k^{CO2}} HCOO^- + A_{ox} \quad (6b)$$

Several FDHs are able to catalyze the carbon dioxide reduction, either *in vivo* or *in vitro*. *In vivo*, the most obvious example is provided by the enzymes of acetogens, organisms that fix carbon dioxide reducing it to formate and, eventually, producing acetate [23–27]; *in vitro*, several other FDHs of distinct metabolic pathways were described to be able to catalyze the carbon dioxide reduction under appropriate conditions.

One of the most efficient carbon dioxide reducers so far described is a W-FDH from *Syntrophobacter fumaroxidans*[7]. This bacterium expresses two W-FDHs, both believed to hold an active site selenocysteine residue [138–141]: one (FDH2) is a very efficient formate oxidizer ($k \approx 5.6 \times 10^3\ s^{-1}$ (value reported as 2700 U mg^{-1}); K_m^{HCOO-} of 10 μM), with a 30-fold lower rate of carbon dioxide reduction, $k \approx 0.2 \times 10^3\ s^{-1}$ (reported as 90 U mg^{-1}), which is still a very high value comparatively to other enzymes; but the other *S. fumaroxidans* W-FDH (FDH1) displays an extremely high rate of carbon dioxide reduction, $k \approx 2.5 \times 10^3\ s^{-1}$ (reported as 900 U mg^{-1}; K_m^{CO2} not determined, assays with 10 mM HCO_3^-), with a slightly lower formate oxidation rate ($k \approx 1.9 \times 10^3\ s^{-1}$ (reported as 700 U mg^{-1}); K_m^{HCOO-} of 40 μM). This last enzyme (FDH1) is also a good electrocatalyst to carry out the electrochemical reduction of carbon dioxide to formate, using mild conditions and applying small overpotentials, with a maximum current density of ≈80 μA cm^{-2} that corresponds to $k \approx 110\ s^{-1}$ (from a monolayer of enzyme) [142]. Intriguingly, while in homogeneous catalysis in solution the carbon dioxide reduction is slightly faster than the formate oxidation, in the electrochemical-assisted reduction/oxidation is the formate oxidation that is more than 2 times faster (with a current density of ≈200 μA cm^{-2} [142]). Even so, this W-FDH is so far the fastest carbon dioxide reducer described.

The *Acetobacterium woodii* developed another very interesting FDH (Mo-FDH, thought to hold an active site selenocysteine), which takes part in a remarkable hydrogen-dependent carbon dioxide reductase complex that couples the carbon dioxide reduction directly to the dihydrogen oxidation [143,144]. This complex catalyzes the carbon dioxide reduction with a k_{cat} of 28 s^{-1} (reported as 10 U/mg; K_m^{HCO3-} of 37 mM), displaying only slightly higher rates of formate oxidation (reported as 14 U/mg; K_m^{HCOO-} of 1 mM) [143]. Also whole cells of *A. woodii* were shown to act as catalysts, producing formate from carbon dioxide and dihydrogen. Hence, a whole-cell biocatalysis of carbon dioxide hydrogenation by *A. woodii* can be envisaged to store dihydrogen/produce formate [143,145].

FDHs from other acetogenic organisms were shown to also reduce carbon dioxide, but at considerably lower rates [146,147]. The NAD-dependent W-FDH (selenocysteine-containing) from *Clostridium carboxidivorans*, *e.g.*, displays a k_{cat} of only 0.08 s^{-1} (K_m^{HCO3-} of 50 μM) [147–149]. The occurrence of this thermodynamically highly unfavorable reaction (reduction potential of $NAD(P)^+/NAD(P)H$ of −0.32 V, *versus* −0.43 V for $CO_2/HCOO^-$) highlights the key role played by these enzymes and their redox-active centers in overcoming the reaction energy barrier and allowing the organisms to reduce carbon dioxide to formate.

The same remarkable situation is found in other organisms that developed Mo-FDHs dependent on NAD. This is exemplified by the *Cupriavidus oxalaticus* (cysteine-containing) enzyme, which is known for long to be able to reduce carbon dioxide, but with a k_{cat} of ≈3 s^{-1}, ≈30 times lower than the one for formate oxidation (K_m^{HCO3-} of 40 mM; K_m^{HCOO-} of 100 μM) [150], or by the *R. capsulatus* (cysteine-containing) enzyme that has a k_{cat} of 1.5 s^{-1}, ≈25 times lower than the one for formate oxidation (K_m^{HCOO-} of ≈280 μM; $K_{m\ CO2}$ not determined, assays with 100 mM HCO_3^-) [84].

[6] *In vivo*, in the majority of the cases, the reactions are tuned to operate only in one direction; this is determined by the reduction potential of the enzyme redox centers, by the available physiological electron partners and substrates and by the redox status of the subcellular location where the reaction takes place. The exceptions are mainly concerned with regulation points of the metabolism.

[7] In syntrophic cultures of acetogenic and hydrogenotrophic methanogenic organisms, the dihydrogen and formate produced by acetogens are consumed by the methanogens [129–135]. In this way, biological polymers can be converted into methane and carbon dioxide, in a global process where dihydrogen and formate are important interspecies mediators of reducing equivalents [136–137]. The syntrophic bacterium *Syntrophobacter fumaroxidans* can oxidize propionate to acetate and use the reducing equivalents to reduce protons to dihydrogen or carbon dioxide to formate. These products are then transferred to a syntrophic partner, *Methanospirillum hungatei*, that acts as a formate and dihydrogen scavenger, in an interspecies formate/dihydrogen transfer [138,139].

The *E. coli* FDH H was as well shown to be a catalyst (in solution) and electrocatalyst of carbon dioxide reduction [151], following an *old* (of 1936) suggestion that this enzyme is able to reduce carbon dioxide to formate in the presence of dihydrogen [152]. Using protein film voltammetry, it was demonstrated that FDH H can catalyze the carbon dioxide reduction with rates only ≈45% of the formate oxidation ones (reported as 80 *versus* 180 μA cm^{-2}) [151]. In contrast, in solution, the carbon dioxide reduction was found to be considerably lower than the formate oxidation (<1 *versus* 160 s^{-1}) [151].

The enzymes from sulfate-reducing bacteria are also very interesting systems to carry out the carbon dioxide reduction. The *Desulfovibrio vulgaris* Hildenborough contains several FDHs and one Mo-FDH (selenocysteine-containing) is able to catalyze the carbon dioxide reduction with a rate of ≈3.4 s^{-1} (reported as 1 U mg^{-1}), even though at lower rates than the ones of formate oxidation (k_{cat} of 262 s^{-1}; $K_m^{HCOO^-}$ of 8 μM) [49]. Nevertheless, the preferential activity of *D. vulgaris* FDHs towards formate oxidation is also very interesting and it can be the basis for a good biocatalyst for converting formate into dihydrogen [153]. In fact, it was very recently demonstrated that *D. vulgaris* growth can be coupled with dihydrogen production from formate (in the absence of sulfate or a syntrophic partner) [153].

Comparatively, the *D. desulfuricans* Mo-FDH (selenocysteine-containing) is a strikingly efficient carbon dioxide reducer. The *D. desulfuricans* enzyme displays a high k_{cat} of ≈50 s^{-1} (K_m^{CO2} of ≈15 μM) [55]. Although the formate oxidation is ≈11 times faster (k_{cat} of ≈550 s^{-1}; K_m of ≈55 μM), the FDH specificity for formate is only ≈3 times higher than the specificity for carbon dioxide (10 *versus* 3.3 μM^{-1} s^{-1}), what favors the reduction of carbon dioxide when formate is present in lower concentrations [55].

From the examples described, it is evident that the carbon dioxide reduction activity of FDHs is wide ranged, going from fast reducers to poor ones. As far as is presently known, a trend cannot be yet assumed. It was initially suggested that the W-FDHs would be better catalysts than the molybdenum counterparts. In fact, the tungstoenzymes, contrary to the molybdoenzymes, are thought to be involved in anaerobic low reduction potential reactions (as the carbon dioxide reduction one), as is supported by the chemical properties of tungsten compounds that exhibit lower reduction potentials, higher bond strengths and higher sensitivity to dioxygen, comparatively to iso-structural molybdenum counterparts [25,27,66,154–159]. This reasoning is perfectly exemplified by the *S. fumaroxidans* W-FDH, with its carbon dioxide reduction rate of ≈2.5×10^3 s^{-1}. Yet, this *ultra-fast* carbon dioxide reduction catalysis is, as far as is presently known, an exception and other W-FDHs display considerably lower rates of carbon dioxide reduction. Conversely, and in spite of the chemical-based prediction, different Mo-FDHs have been described to be able to catalyze the carbon dioxide reduction with appreciable rates, of 1–50 s^{-1}. It is possible that, instead of the metal nature, it is the number of redox-active centers of the enzyme/enzymatic complex that is decisive, as they can act as an "electron capacitor" that ensures the electron supply needed to maintain the active site reduced.

4. Outlook

To activate carbon dioxide is, undoubtedly, a difficult task. The widespread, successful, D-ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBbisCO), *e.g.*, fixes carbon dioxide in a slow (<10 s^{-1} [160]) and promiscuous, energy-wasting (CO_2 *versus* O_2), reaction. Under this scenario of common "catalytic inefficiency", FDHs, or whole-cells systems, are very interesting targets for biotechnological applications of carbon dioxide utilization and/or formate production. These enzymes have the advantage, over present chemical catalysts, of being specific, yielding only one product (formate) and working as homogeneous catalysts. They allow the consumption of the abundant atmospheric carbon dioxide, mitigating environmental problems, with the specific formation a "storable" form of energy (formate/hydrogen). The present major challenges for such a biotechnological application of FDHs concern their oxygen sensitivity, protein long term stability, and slower rates and lower affinities comparative to the reverse formate reduction reaction. The catalytic rates of carbon dioxide reduction would also have to be increased to justify its employment on an industrial scale. Yet, the knowledge acquired in the study of these enzymes (understanding mechanistic strategies and identification of key chemical features) can be explored to develop synthetic, bio-inspired, new catalysts at a long term.

Acknowledgments

This work was supported by the Unidade de Ciências Biomoleculares Aplicadas-UCIBIO which is financed by national funds from FCT/MEC (UID/Multi/04378/2013) and co-financed by the ERDF under the PT2020 Partnership Agreement (POCI-01-0145-FEDER-007728). LBM thanks Fundacão para a Ciência e a Tecnologia, MEC, for a fellowship grant (SFRH/BPD/111404/2015, which is financed by national funds and co-financed by FSE).

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