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Molybdenum-containing CO dehydrogenase and formate dehydrogenases

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Abstract

The molybdenum-containing CO dehydrogenase and the formate dehydrogenases catalyze important interconversions of one-carbon compounds, the former oxidizing CO to CO₂, and the latter the reversible interconversion of CO₂ and formate. Methodologies to study these two enzymes are discussed.



1. Overview

Bacterial interconversion of CO, CO₂ and formate plays an important role in the global carbon cycle. Oxidation of CO to CO₂ is catalyzed by two quite different types of CO dehydrogenase. Obligate anaerobes such as *Moorella thermoacetica* (formerly *Clostridium thermoaceticum*) possess a

nickel- and iron-containing CO dehydrogenase that reversibly reduces CO_2 to CO, which is subsequently condensed with methylcobalamin from acetyl-CoA synthase to form acetyl-CoA in the course of acetogenesis. The enzyme is extremely air-sensitive. The reverse process catalyzes the final step in methanogenesis. On the other hand, aerobes such as *Oligotropha carboxidovorans* possess a molybdenum- and copper-containing CO dehydrogenase that oxidizes CO to CO_2 . In contrast to the clostridial enzyme, the *O. carboxidovorans* enzyme is quite tolerant of O_2 . It is the air-stable CO dehydrogenase that is considered here.

Similarly, there are two broad types of formate dehydrogenase. A variety of organisms, including humans, possess an enzyme that is devoid of redox-active cofactors and catalyzes the oxidation of formate to CO_2 . These enzymes use NAD^+ as the electron acceptor in the course of the reaction, which proceeds via direct hydride transfer within a ternary enzyme•formate• NAD^+ ternary complex. On the other hand, a number of bacteria and archaea possess one or more molybdenum- (or occasionally tungsten) containing formate dehydrogenases that catalyzes the reversible interconversion of formate and CO_2 . It is these latter enzymes that again constitute the focus of the present account.

The interconversions of CO, CO_2 and formate are also important as feedstock compounds for chemical synthesis and, in the case of the interconversion of CO_2 and formate, have enormous potential for the reversible storage of renewable energy (Appel et al., 2013). What follows is a discussion of considerations that bear in studies of the molybdenum-containing CO dehydrogenase and formate dehydrogenases.



2. CO Dehydrogenase

2.1 Introduction

Oligotropha carboxidovorans and related organisms are able to grow aerobically with CO as sole source of both carbon and energy, and in the presence of O_2 (Meyer et al., 1993). The overall stoichiometry involves the oxidation of approximately six equivalents of CO to CO_2 generating the reducing equivalents that provide the energy necessary to fix one equivalent of CO_2 via the pentose phosphate pathway (Jacobitz and Meyer, 1989). The CO dehydrogenase responsible for this oxidation possesses a binuclear molybdenum- and copper-containing active site, the reducing equivalents thus obtained ultimately being passed into the intramembrane

quinone pool and on to a CO-insensitive terminal oxidase (Cypionka and Meyer, 1983). It has been estimated that this process remediates as much as 2×10^8 metric tons of environmental CO each year (Moxley and Smith, 1998; Moersdorf et al., 1992; Jaffe, 1970).

2.2 Genetic context and protein structure

The *O. carboxidovorans* CO dehydrogenase is encoded by a gene cluster encoding twelve distinct proteins (Kraut et al., 1989; Kang and Kim, 1999; Santiago et al., 1999). The structural genes are *coxMSL*, which form the native $(\alpha\beta\gamma)_2$ enzyme. Both CoxF and CoxI are homologous to the *E. coli* XdhC protein that inserts a terminal sulfur group into the molybdenum coordination sphere (Matthies et al., 2004; Neumann et al., 2006; Schumann et al., 2008). CoxD exhibits homology to known membrane-integral ATPases and appears to be involved in incorporation of copper into the maturing binuclear center (Pelzmann et al., 2009). CoxB, CoxC, CoxH and CoxK are predicted to possess multiple transmembrane helices and are likely involved in anchoring CO dehydrogenase on the inner side of the cytoplasmic membrane, where quinones become reduced as the enzyme oxidizes CO (Cypionka and Meyer, 1983; Spreitler et al., 2010).

The demand for carbon necessary to maintain cell growth ensures that *O. carboxidovorans* and related organisms accumulate large amounts of intracellular CO dehydrogenase when grown on CO and it is straightforward to purify the wild-type protein in relatively high quantities (Meyer and Schlegel, 1980; Zhang et al., 2010). CO dehydrogenases from both *O. carboxidovorans* (Dobbek et al., 1999) and *Hydrogenophaga pseudoflava* (Hanzelmann et al., 2000) have been crystallographically characterized and have very similar structures. Fig. 1 shows structure of the *O. carboxidovorans* enzyme, with the 18 kDa CoxS subunit in red containing two [2Fe-2S] iron-sulfur clusters, the 30 kDa CoxM subunit with one equivalent of FAD in yellow, and the 89 kDa CoxL subunit containing the active site Mo/Cu binuclear center in gray. Each subunit has considerable homology to the corresponding parts of other members of the xanthine oxidase family of molybdenum-containing enzymes, the unique nature of the binuclear active site of CO dehydrogenase notwithstanding (Hille, 1996a; Hille et al., 2014a). The structure of the active site binuclear center of CO dehydrogenase in its oxidized form is also shown in Fig. 1 (inset) (Dobbek et al., 2002; Meyer-Klaucke et al., 2001). The oxidized Mo(VI) ion has the distorted square-pyramidal coordination geometry seen in other members of the xanthine oxidase family of molybdenum-containing enzymes, with

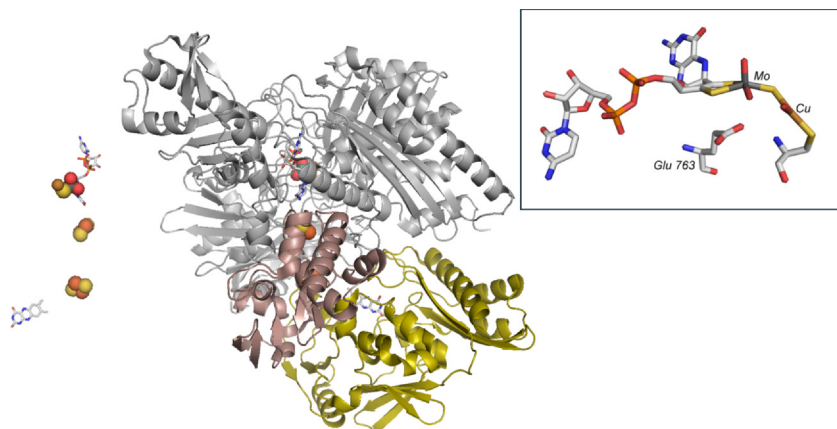


Fig. 1 The structure of the *O. carboxidovorans* CO dehydrogenase (PDB 1N5W). One protomer of the dimeric protein is shown, with the CoxS subunit in red, the CoxM subunit in yellow and the CoxL subunit in gray. In the protomer on the left, the polypeptide has been deleted to show the disposition of the binuclear Mo/Cu center of CoxL, top, the two [2Fe-2S] of CoxS, middle, and the isoalloxazine of the FAD in CoxM, bottom. The inset shows a close-up of the binuclear cluster in the active site.

an apical Mo=O and an equatorial plane consisting of a second Mo=O group rather than the catalytically labile Mo-OH seen in other family members (Zhang et al., 2010) and two sulfurs from the pyranopterin cofactor (present as the dinucleotide of cytosine) that is common to all molybdenum and tungsten enzymes (other than nitrogenase). The final ligand to the molybdenum is a μ -sulfido bridge to the Cu(I) in place of the Mo=S found in other members of this family that is presumably inserted by CoxF and/or CoxI; the Cu(I) ion is also liganded by Cys 388 and a water molecule. Steric access to the deeply buried binuclear center of CO dehydrogenase is quite restricted, and in particular Phe 390 of CO dehydrogenase (equivalent to Phe 914 in the bovine xanthine oxidoreductase which constitutes part of the substrate binding site) is displaced into what would otherwise be the solvent access channel in order to accommodate the copper and Cys 388 that coordinates it. Still, access is sufficient as to allow not only CO but even larger molecules such as *n*-butylisonitrile (Dobbek et al., 2002), a CO analog.

A heterologously expressed *O. carboxidovorans* CO dehydrogenase in *E. coli* exhibits spectroscopic and kinetic properties comparable to the native enzyme (Kaufmann et al., 2018), and a preliminary study of several variants of active site residues has been undertaken, indicating that mutations at Glu 763,

Phe 390 and Cys 388 all abolished activity. Unfortunately, these variants possessed only low levels of the pyranopterin cofactor, indicating it was likely that loss of activity was trivially due to lack of incorporation of the Mo/Cu center. Several thiol-containing compounds, including cysteine, mercaptoethanol and dithiothreitol, reversibly inhibit CO dehydrogenase with modest K_i 's in the mM range (Kress et al., 2014). Extended X-ray absorption fine structure analysis of the copper environment in mercaptoethanol-complexed CO dehydrogenase suggests direct binding of the copper, presumably displacing the water molecule that is otherwise bound to the copper (Kress et al., 2014). CO dehydrogenase is neither inhibited nor inactivated by O_2 , but the reduced enzyme is readily reoxidized by O_2 and experiments with the reduced protein are routinely done anaerobically under an atmosphere of N_2 or Ar.

2.3 Mechanistic studies of the binuclear Mo/Cu center

In the course of reaction of CO dehydrogenase with CO, an EPR signal due to the partially reduced Mo/Cu binuclear center (formally Mo^V/Cu^I) accumulates that exhibits extremely strong coupling to the copper ion (Zhang et al., 2010), as shown in Fig. 2. The signal is unchanged on preparation of the sample in D_2O , indicating no strong proton coupling, but modest line-broadening is seen when ^{13}CO is used as substrate, reflecting weak coupling to the substrate carbon (Zhang et al., 2010). A Mo^V/Cu^I model for the EPR-active form of the binuclear active site of CO dehydrogenase having a $Mo^VO-\mu S-Cu^I$ core exhibits very similar copper hyperfine coupling (Gourlay et al., 2006) that reflects substantial delocalization of unpaired spin density on the copper, despite it being formally Cu^I . ENDOR work with ^{13}CO -reduced CO dehydrogenase shows essentially isotropic ^{13}C hyperfine coupling, indicating that at least two atoms intervene between the molybdenum and carbon (Shanmugam et al., 2013). The signal has been interpreted to arise from a Mo^V/Cu^I species.

having CO bound at the copper of the binuclear center, displacing the water seen in the X-ray crystal structure. Backbonding from the copper CO weakens the C-O bond, thereby activating it for nucleophilic attack upon coordinating to the copper.

A high-resolution (1.1 Å) X-ray crystal structure of CO dehydrogenase with the CO analog *n*-butylisocyanide shows the inhibitor inserted between the Mo and Cu, rupturing the bridging S-Cu bond (Dobbek et al., 2002). A reaction mechanism has been proposed in which CO similarly inserts into the binuclear center to yield a bridging thiocarbamate concomitant with

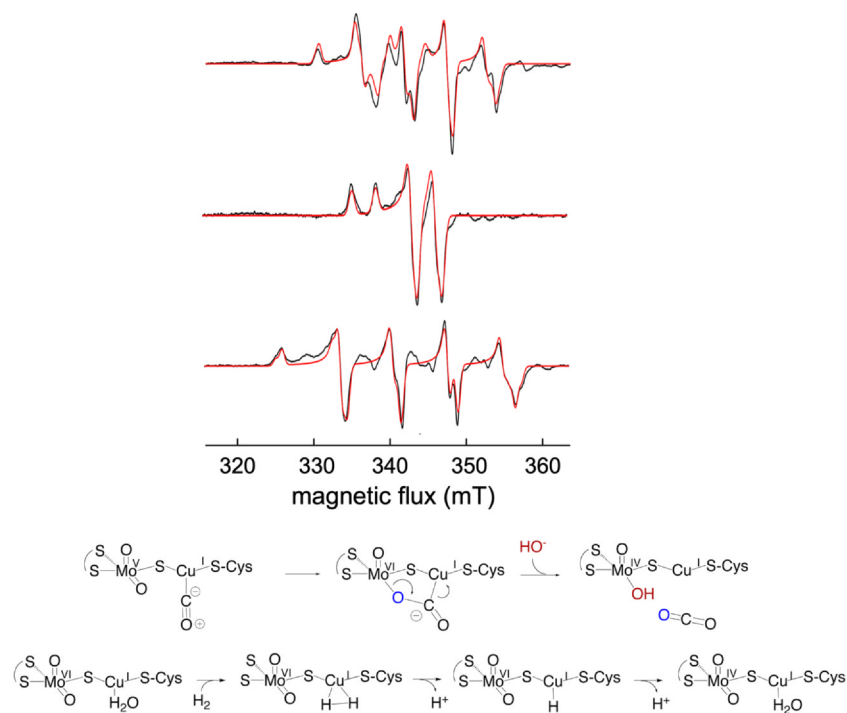


Fig. 2 EPR signals and reaction mechanisms for CO dehydrogenase. Top, EPR signals exhibited by the binuclear center of native CO dehydrogenase on reduction with CO, of the silver-substituted enzyme reduced by CO and the native enzyme reduced by H₂. Spectra are in black and simulations in red using the following parameters. Spectra are, from top to bottom: native enzyme reduced with CO, $g_{1,2,3} = 2.0010, 1.9604, 1.9549$ and $A_{1,2,3}({}^{63,65}\text{Cu}) = 117, 164, 132$ MHz; silver-substituted enzyme reduced by CO ($g_{1,2,3} = 2.0043, 1.9595, 1.9540$ and $A_{1,2,3}({}^{63,65}\text{Cu}) = 82.0, 78.9, 81.9$ MHz); and native enzyme reduced with H₂ ($g_{1,2,3} = 2.0127, 1.9676, 1.9594$, $A_{1,2,3}({}^{63,65}\text{Cu}) = 169, 200, 170$ MHz and $2 \times A_{1,2,3}({}^1\text{H}) = 80, 20, 130$ MHz). Shown below are the proposed mechanisms for CO oxidation (upper) and H₂ oxidation (lower) as catalyzed by CO dehydrogenase.

reduction of the molybdenum, the thiocarbamate hydrolyzing to regenerate the sulfur bridge to complete the catalytic cycle. The reaction has also been examined computationally (Siegbahn and Shestakov, 2005; Hofmann et al., 2005). One group has concluded that a very stable thiocarbamate intermediate such as that proposed on the basis of the structure of the isonitrile-complexed enzyme indeed forms and passes over a relatively low-lying transition state to release CO₂ as water/hydroxide displaces the thiocarbamate from the binuclear cluster (Siegbahn and Shestakov, 2005). A second group has concluded that such a thiocarbamate intermediate represents a

thermodynamic trap and is not likely to form in the course of catalysis; the reaction instead is proposed to proceed through a series of three intermediates that involve the CO carbon bonded to each of the three atoms (Mo, μ S and Cu) that contribute to the highly delocalized redox-active orbital of the oxidized binuclear center (Hofmann et al., 2005). It has been suggested these might in fact be better thought of as alternate resonance representations of a single structure, or alternatively discrete, rapidly interconverting intermediates (Shanmugam et al., 2013). Assuming that the equatorial Mo=O of CO dehydrogenase is in fact catalytically labile, as seen in xanthine oxidase (Hille and Sprecher, 1987), the binuclear cluster appears to be constructed so as to provide a substrate binding site for CO adjacent to the molybdenum that positions the CO appropriately for attack by the equatorial Mo=O and spatially extend the formally redox-active Mo d_{xy} orbital such that it can accept an electron pair in the course of the reaction at the more remote (copper) site as shown in Fig. 2.

Like several of the Ni/Fe CO dehydrogenases (Can et al., 2014), the Mo/Cu enzyme is reduced by H₂ (Zhang et al., 2010). A new EPR signal arising from the binuclear center is seen in the course of the reaction that exhibits coupling to two approximately equivalent and solvent-exchangeable protons, in addition to the strong copper hyperfine, as shown in Fig. 2. By analogy to the binding of CO to the Cu of the binuclear center, the signal-giving species is proposed to possess H₂ coordinated (in a side-on η_2 fashion) to the copper of a partially reduced binuclear center (Wilcoxon and Hille, 2013), for which chemical precedent exists (Wang et al., 2013; Frohman et al., 2013). The reaction is thought to proceed by reversible deprotonation of the polarized H₂ to yield a hydride complex (for which chemical precedent also exists (Hulley et al., 2013)), with a second (also reversible) deprotonation resulting in population of the redox-active orbital, as shown in Fig. 2. This mechanism predicts that the enzyme catalyzes exchange of deuterium from D₂O into H₂, but this has not yet been demonstrated.

2.4 Reaction with quinones

Physiologically, CO dehydrogenase passes the reducing equivalents obtained from oxidation of CO into the intramembrane ubiquinone pool via its FAD site, and several quinone species are in fact able to effectively reoxidize dithionite-reduced CO dehydrogenase (Wilcoxon et al., 2011b). Ubiquinone itself reacts with a second-order rate constant of $2.99 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, while 1, 4-benzoquinone exhibits hyperbolic dependence on substrate concentration,

with a k_{ox}/K_d of $2.13 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. On the basis of these numbers, it can be concluded that the rate-limiting step for overall turnover resides in the reductive half-reaction.

2.5 Experimental considerations

Growth of any organism on CO as sole carbon source requires attention to safety. In the author's laboratory, a 20 L fermentor is placed in a floor-to-ceiling hood in a small (~ 120 sq.ft.) room equipped with a CO sensor. After growth to late log phase, the culture is transferred to sealed, 1 L centrifuge bottles within the hood, and the cells then spun down. The supernatant is left in the hood overnight to degas, then disposed of. Harvested cells are handled in a conventional hood on ice, being treated with a solution containing both phenylmethylsulfonylfluoride to inhibit proteases and DNase to minimize viscosity prior to lysis using a French press. CODH is quite air-stable, and no extraordinary measures are required during purification to minimize exposure to O_2 . Although membrane-associated in the cell, it is not membrane-integral and is readily soluble up to concentrations of 1 mM or higher. Protein purification (Zhang et al., 2010) involves standard FPLC steps involving Q-Sephacryl and Sephacryl S-300 chromatography using a published protocol. Enzyme purity can be gauged by a ratio of absorbance at 450 nm to that at 280 nm of ~ 5.5 .

The bridging sulfur and copper of the binuclear center of CO dehydrogenase can be removed by treatment of the enzyme with cyanide, and a protocol has been developed for reconstituting the binuclear center by successive treatment of the apoenzyme with sulfide and $\text{Cu(I)}\cdot\text{thiourea}$ (Resch et al., 2005). Interestingly, when $\text{Ag(I)}\cdot\text{thiourea}$ is used instead, activity is partially recovered (Wilcoxon et al., 2011a) and the enzyme thus reactivated can be fully reduced by CO, although at a fivefold slower rate than seen with native enzyme (8 s^{-1} as opposed to 51 s^{-1}). The EPR signal seen upon partial reduction of the silver-substituted enzyme by CO (Fig. 2) shows the doublets expected for substitution of Ag for Cu. The reduced activity of the silver-substituted enzyme can be rationalized as arising from the lower π -backbonding capacity of silver relative to copper, as weaker backbonding is expected to result in less weakening of the C-O bond of bound substrate and a higher activation barrier to reaction. On the basis of the relative rate constants for reduction of the copper- and silver-containing enzyme, the weaker backbonding in the silver-substituted enzyme reflects $\sim 1 \text{ kcal/mol}$ in compromised transition state stabilization upon substitution of silver for copper.



3. Formate dehydrogenases

3.1 Introduction

Enzymes that oxidize formate to CO_2 are broadly distributed and come in two quite different varieties: simple metal-independent enzymes that catalyze a direct hydride transfer from formate to NAD^+ via a ternary $\text{E}\cdot\text{formate}\cdot\text{NAD}^+$ complex, and metal-dependent enzymes containing molybdenum or tungsten that are frequently components of quite large assemblies. These latter enzymes operate via a ping-pong mechanism in which the molybdenum (or tungsten) center is reduced by formate, with the reducing equivalents thus obtained subsequently transferred to other redox-active centers in the system and eventually to an oxidizing substrate. While most metal-containing formate dehydrogenases function physiologically in the direction of formate oxidation to CO_2 , at least some work has focused on the reaction in the opposite direction with the enzyme functioning as a CO_2 reductase, taking reducing equivalents from a source such as H_2 and using them to reduce CO_2 to formate in a reaction of considerable interest for bioremediation of atmospheric CO_2 and the energy economy. Despite a considerable literature devoted to whether a given formate dehydrogenase is or is not able to catalyze the reverse reaction, the reduction of CO_2 to formate, the most recent work strongly suggests that all these metal-containing enzymes are fully reversible when care is taken to use CO_2 rather than bicarbonate as substrate (the bicarbonate/ CO_2 equilibrium in aqueous solution being relatively slow compared to the typical rates of enzyme turnover) (Yu et al., 2017). Although not considered further here, it is worth noting that the Mo- and/or W-containing subunits of formate dehydrogenases have considerable structural and functional homology to the corresponding subunits of formylmethanofuran dehydrogenases from methanogenic archaea (Thauer et al., 2008; Wagner et al., 2016). Indeed many organisms encode separate but very similar (in some cases virtually identical) formate dehydrogenases containing tungsten on the one hand or molybdenum on the other; the same is true for formylmethanofuran dehydrogenases.

3.2 Systematics

Whereas the CO dehydrogenase discussed above is a member of the xanthine oxidase family of molybdenum-containing enzymes, the molybdenum-containing formate dehydrogenases are members of the DMSO reductase family (Hille, 1996b; Hille et al., 2014b). Several formate dehydrogenases have been

characterized crystallographically (Boyington et al., 1997; Raaijmakers et al., 2002; Jormakka et al., 2002; Oliveira et al., 2020) or by cryoelectron microscopy (Radon et al., 2020), and found to possess very similar overall folds. All contain a metal center coordinated to two equivalents of a pyranopterin-containing cofactor that chelates the metal via an enedithiolate side chain, as shown in Fig. 3; this cofactor is elaborated as the dinucleotide of guanine in all cases. The remainder of the metal coordination sphere is.

made up of a cysteine or selenocysteine residue provided by the polypeptide and a terminal sulfido ligand that is essential for catalytic activity (Thome et al., 2012). The *E. coli* FdhD gene product is a sulfur transferase that, in conjunction with the IscS cysteine desulfurase, catalyzes the insertion of this Mo=S ligand (Arnoux et al., 2015). In addition, most formate dehydrogenases have at least one, and as many as seven, iron-sulfur cluster that lies in close proximity to the molybdenum center.

The metal-containing formate dehydrogenases can be categorized on the basis of the metal present in the active site, molybdenum or tungsten, and whether the protein ligand to the metal is cysteine or selenocysteine. A final level of variation in these enzymes, as discussed further below, is their overall subunit architecture, which ranges from the simple, monomeric FdhF formate dehydrogenases from, e.g., *E. coli* (containing molybdenum and a single [4Fe-4S] cluster) (Boyington et al., 1997) to the cytosolic FdsDABG formate dehydrogenase from *Cupriavidus necator* or *Rhodobacter capsulatus*, containing seven iron-sulfur clusters and FMN in addition to its molybdenum center (Radon et al., 2020).

Mo-containing FdhF formate dehydrogenases from *E. coli* (with selenocysteine coordinated to the metal) (Axley et al., 1990a) or *Pectobacterium atrosepticum* (with cysteine) (Finney et al., 2019) are cytosolic components of membrane-integral formate:hydrogen lyases, fully reversible systems that either generate or consume H₂ depending on the growth conditions (Finney and Sargent, 2019). The W-containing Fdh1AB from *Methylobacterium extorquens* (containing cysteine) and the Mo-containing FdhABC from organisms such as *Desulfovibrio vulgaris* (containing selenocysteine) (Oliveira et al., 2020) are periplasmically localized. The FdnGHI and FdoGHI formate dehydrogenases from, e.g., *E. coli*, are ($\alpha\beta\gamma$)₃ trimers possessing Mo-containing subunits resembling the above FdhF proteins that are attached to membrane-integral, heme-containing subunits via a subunit containing four [4Fe-4S] clusters (Jormakka et al., 2002). FdnGHI is expressed under strictly anaerobic conditions, while FdoGHI is expressed

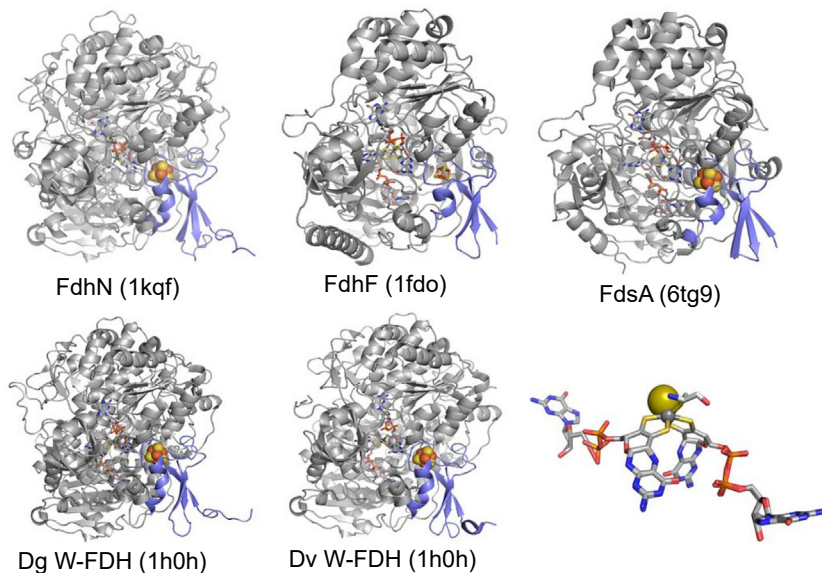


Fig. 3 Representative structures of the metal-containing domains/subunits of formate dehydrogenases. Top, from left to right: the molybdenum-containing FdhN formate dehydrogenase from *E. coli*, the FdhF formate dehydrogenase from *E. coli*; the C-terminal portion of FdsA from the FdsDABG formate dehydrogenase of *R. capsulatus*. Bottom: The tungsten-containing subunits of the formate dehydrogenases from *D. gigas* (left) and *D. vulgaris* (right). In all five cases, the domain containing a [4Fe-4S] cluster is shown in blue. The overall structural homologies among the five structures are evident. The PDB accession numbers used to generate these images are given. At bottom right is a close-up of the active site molybdenum center in the *R. capsulatus* C-terminal domain of FdsA.

in the transition from aerobic to anaerobic growth; both contain selenocysteine. The NAD^+ -dependent FdsDABG enzymes from *Cupriavidus necator* (Friedebold and Bowien, 1993) and *Rhodobacter capsulatus* (Hartmann and Leimkuehler, 2013) are cytosolic and organized as $(\alpha\beta\gamma)_2$ dimers of trimers. More complicated still is the cytosolic H_2 -dependent CO_2 reductase from, e.g., *Acetobacterium woodii* consisting of an iron-only hydrogenase subunit, two different subunits containing four [4Fe-4S] clusters, and an FdhF-like molybdenum- and [4Fe-4S]-containing subunit (Schuchmann and Mueller, 2013). Interestingly, the *A. woodii* genome encodes tandem genes for this last subunit, one encoding a subunit that has selenocysteine coordinated to the molybdenum and the other cysteine; it is presently not clear under what conditions each can be functionally incorporated.

Despite the considerable diversity in overall subunit organization and architecture, the protein folds of the Mo- and W-containing subunits of formate dehydrogenases are remarkably similar. Fig. 3 shows the Mo- or W-containing subunits (or domain in the case of FdsDABG) for the formate dehydrogenases that have to date been structurally characterized: the *E. coli* FdhF formate dehydrogenase (Mo, SeCys) (Boyington et al., 1997), the FdnG subunit of the *E. coli* FdnGHI formate dehydrogenase (Mo, SeCys) (Jormakka et al., 2002), the FdhA subunit of the *D. gigas* FdhAB formate dehydrogenase (W, SeCys) (Oliveira et al., 2020) and the C-terminal domain of FdsA from the *R. capsulatus* FdsDABG enzyme (Radon et al., 2020). The overall folds of the proteins are clearly very similar, including the position of the [4Fe-4S] cluster present in each of these subunits. Also highly conserved are two additional active site residues, a His immediately to the C-terminal side of the Cys/SeCys that coordinates the metal, and an Arg that constitutes part of the substrate binding site. A close-up of the active site of the *E. coli* FdhF formate dehydrogenase is shown in Fig. 4.

Formate dehydrogenases are thought to be evolutionarily ancient, tracing back to the Last Universal Common Ancestor to all extant life forms. Both the acetogenic Wood-Ljungdahl pathway of bacteria and the version found in methanogenic archaea begin with CO₂ reduction by a Mo- or W-containing enzyme (an H₂-oxidizing CO₂ reductase in the former case, a formylmethanofuran dehydrogenase in the latter) (Ragsdale and Pierce, 2008). The two branches share many common intermediates, including sequential formyl-, methenyl-, methylenyl- and methylpterin species.

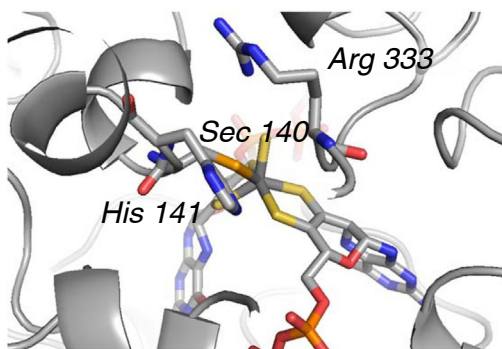


Fig. 4 The active site of the *E. coli* FdhF formate dehydrogenase. Shown are the highly conserved His 141 and Arg 333, as well as the molybdenum-coordinating Sec 140.

3.3 Reaction mechanism

Given the overall similarities in active site structures, it is likely that all molybdenum- (and tungsten)-containing formate dehydrogenases act via fundamentally the same reaction mechanism regardless of whether they possess Cys or SeCys coordinated to the metal, and over the years various mechanisms have been proposed for one or another enzyme (Khangulov et al., 1998; Leopoldini et al., 2008; Mota et al., 2011; Maia et al., 2016). A key mechanistic clue was the demonstration that oxygen from solvent was not incorporated into product CO_2 in the course of the reaction (Khangulov et al., 1998). Although the observation that oxygen from solvent is not incorporated into product CO_2 has recently been called into question (Kumar et al., 2023), the most recent work has clearly demonstrated that oxygen derived from solvent is not incorporated into product CO_2 (D. Niks, R. Hille and coworkers, *J. Am. Chem. Soc.*, in press).

With both the SeCys-containing *E. coli* FdhF (Khangulov et al., 1998) and Cys-containing *C. necator* FdsABG (Niks et al., 2016; Niks and Hille, 2019), the C_α hydrogen of formate is transferred to the molybdenum center in the course of formate oxidation and is strongly coupled in the EPR signal of the Mo(V) oxidation state. It has been proposed that the hydrogen is present as a Mo-SH group (Niks et al., 2016), consistent with the similarly strong hyperfine coupling of Mo-SH protons that is observed in both model complexes (Wilson et al., 1991; Young and Wedd, 1997) and other enzymes (Malthouse et al., 1981). It has been concluded that the reaction proceeds as a simple hydride transfer to give the fully reduced Mo (IV) species, with the EPR-detectable Mo(V) valence state formed upon subsequent transfer of one electron out of the molybdenum to other redox-active centers in the protein, as shown in Fig. 5. In support of this mechanism, formate is known to be a good hydride donor (the reduction of NAD^+ by the metal-free formate dehydrogenases necessarily occurs via hydride transfer) and the $\text{Mo}=\text{S}$ group of other molybdenum-containing enzymes (notably xanthine oxidase (Xia et al., 1997), 1999) is known to be a good hydride acceptor. An important implication of this mechanism is

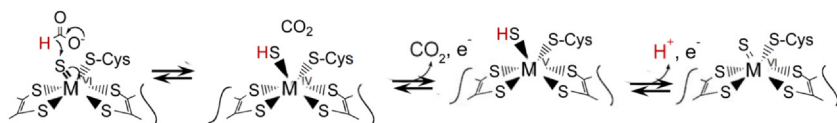


Fig. 5 A simple, fully reversible hydride transfer mechanism for the metal-containing formate dehydrogenases. After Niks et al. (2016).

that the molybdenum coordination sphere is coordination stable, remaining six-coordinate throughout the catalytic sequence with no dissociation of either the Mo=S or Mo-(Se)Cys ligands to the metal.

The hydride transfer mechanism has received wide, if not universal, acceptance, but it has recently been suggested that the reaction proceeds via sequential one-electron transfers rather than a single hydride transfer step (Nazemi et al., 2022; Liu et al., 2022). This seems unlikely on the basis of work with xanthine oxidase, another enzyme in which a Mo=S group functions as a hydride acceptor, where it has been shown unambiguously that there is no correlation between the one-electron reduction potential for a wide series of substrates (determined radiolytically) with either $\log(k_{\text{red}})$ or $\log(k_{\text{red}}/K_d)$ from rapid-reaction studies, indicating that the reaction does proceed in sequential one-electron steps (Stockert et al., 2002).

3.4 Experimental considerations

Selenocysteine- and/or tungsten-containing formate dehydrogenases are generally extremely air-sensitive, but some Mo/cysteine enzymes, e.g., the *C. necator* FdsABG enzyme, are less so when 10 mM nitrate, is added as a protectant (Friedebold and Bowien, 1993). O₂ inactivation of FdsDABG has been shown to involve O₂•⁻, and superoxide dismutase has been found to be as effective in retaining activity as nitrate; inhibition appears to involve oxidation of the catalytically essential sulfido group to sulfite (Hakopian et al., 2022). A general assay for the oxidation of formate to CO₂ follows the anaerobic reduction of benzyl or methyl viologen in the presence of formate at pH 7–7.5 (Axley et al., 1990a, 1990b). In addition to the above general activity assay, the NAD⁺-dependent FdsABG activity can be conveniently followed using the absorbance change associated with reduction of NAD⁺ (Niks et al., 2016). Assays in the reverse reaction should use a solution saturated with CO₂ (29.5 mM at standard temperature and pressure), with NADH or dithionite-reduced benzyl/methyl viologen as source of reducing equivalents (Yu et al., 2017).

Both native (Oliveira et al., 2020; Friedebold and Bowien, 1993; Axley et al., 1990b) and recombinant (Hartmann and Leimkuhler, 2013) formate dehydrogenase preparations have been reported. A common issue in both cases has to do with assessing the degree of functionality of the isolated material. Recombinant systems are prone to incomplete incorporation of molybdenum (or tungsten) to varying degrees, but even most native preparations may still have lower than maximal specific activity, frequently due to loss of (or failure to incorporate) the sulfido ligand to the metal that is

known to be catalytically essential (Thome et al., 2012). Maintaining activity can be facilitated by inhibitors such as azide (Axley et al., 1990a), nitrate (Friedebold and Bowien, 1993) or superoxide dismutase (Hakopian et al., 2022). The extent of functionality can be easily estimated by comparing the extent of reduction with formate with that seen by a non-specific reagent such as sodium dithionite, as monitored by UV/visible spectroscopy.



4. Summary

Relatively straightforward procedures for expression and purification of both native and recombinant the CO dehydrogenase from aerobes such as *O. carboxidovorans* and metal-containing formate dehydrogenases from various obligate or facultative anaerobes have been described in the literature. EPR has proven extremely useful in characterizing the molybdenum (or tungsten, in the case of the formate dehydrogenases) centers that constitute their active sites, and their reaction mechanisms are fairly well understood despite the absence of convenient UV–visible spectroscopic probes that directly report on the active site. Still, these enzymes invariably possess one or more additional redox-active centers in addition to the molybdenum center (minimally a [4Fe–4S] cluster adjacent to the active site) and these provide convenient spectral changes that permit the accumulation of reducing equivalents that accumulate in the course of reaction with substrate to be monitored. There is a general, if not universal, agreement as to the reaction mechanism of both types of enzyme, although considerable work remains to elucidate the specific catalytic roles of active site amino acid residues, particularly in the case of the formate dehydrogenases. Not directly related to reaction mechanism, considerable efforts are under way to develop industrially effective systems based on one or another formate dehydrogenase for the interconversion of formate and CO₂, both in remediation of atmospheric CO₂ and the reversible storage of energy from renewable sources.

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