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EPR characterization of the molybdenum(V) forms of formate dehydrogenase from *Desulfovibrio desulfuricans* ATCC 27774 upon formate reduction

María G. Rivas [a], Pablo J. González [a], Carlos D. Brondino [b,*], José J.G. Moura [a], Isabel Moura [a,*]

[a] *REQUIMTE-CQFB, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Monte de Caparica, Portugal*
[b] *Departamento de Física, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, 3000 Santa Fe, Argentina*

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Dedicated to the memory of Edward I. Stiefel in recognition of his large contribution to our knowledge on the bioinorganic chemistry of metalloenzymes

Abstract

The EPR characterization of the molybdenum(V) forms obtained on formate reduction of both as-prepared and inhibited formate dehydrogenase from *Desulfovibrio desulfuricans* ATCC 27774, an enzyme that catalyzes the oxidation of formate to CO_2, is reported. The Mo(V) EPR signal of the as-prepared formate-reduced enzyme is rhombic ($g_{max} = 2.012$, $g_{mid} = 1.996$, $g_{min} = 1.985$) and shows hyperfine coupling with two nuclear species with $I = 1/2$. One of them gives an anisotropic splitting and is not solvent exchangeable ($A_{max} = 11.7$, $A_{mid} = A_{min} =$ non-detectable, A-values in $cm^{-1} \times 10^{-4}$). The second species is exchangeable with solvent and produces a splitting at the three principal g-values ($A_{max} = 7.7$, $A_{mid} = 10.0$, $A_{min} = 9.3$). The hyperfine couplings of the non-solvent and solvent exchangeable nuclei are assigned to the hydrogen atoms of the β-methylene carbon of a selenocysteine and to a Mo ligand whose nature, sulfydryl or hydroxyl, is still in debate. The Mo(V) species obtained in the presence of inhibitors (azide or cyanide) yields a nearly axial EPR signal showing only one detectable splitting given by nuclear species with $I = 1/2$ ($g_{max} = 2.092$, $g_{mid} = 2.000$, $g_{min} = 1.989$, $A_{max} =$ non-detectable, $A_{mid} = A_{min} = 7.0$), which is originated from the α-proton donated by the formate to a proximal ligand of the molybdenum. The possible structures of both paramagnetic molybdenum species (observed upon formate reduction in presence and absence of inhibitors) are discussed in comparison with the available structural information of this enzyme and the structural and EPR properties of the closely related formate dehydrogenase-H from *Escherichia coli*.

Keywords: Formate dehydrogenase; Molybdenum-containing enzymes; Dimethylsulphoxide reductase family; Electron paramagnetic resonance

1. Introduction

Formate dehydrogenase (Fdh) catalyzes the oxidation of formate to carbon dioxide according to the reaction:

$$HCOO^- \rightarrow CO_2 + H^+ + 2e^- \quad E^0 = -420\ mV$$

Until now, the crystal structures of three Fdhs belonging to the DMSO reductase family of molybdo/tungsten-enzymes have been reported: the Mo-containing *Ec* Fdh-H [1,2] and *Ec* Fdh-N [3,4] and the W-containing *Dg* Fdh [5,6]. The catalytic subunit of these three proteins (80–110 kDa) contains the active site and one [4Fe–4S] cluster, and shows an identical polypeptide fold [7]. The oxidized active site [Mo/W(VI)] (Fig. 1a) is coordinated to four sulfur atoms from two pterin ligands (MGD), one selenium atom from a selenocysteine, and a nonprotein ligand labeled as X. Ligand X was identified as a hydroxyl group

* Corresponding authors. Fax: +54 342 4575221 (C.D. Brondino), +351 21 2948550 (I. Moura).
E-mail addresses: brondino@fbcb.unl.edu.ar (C.D. Brondino), isa@dq.fct.unl.pt (I. Moura).

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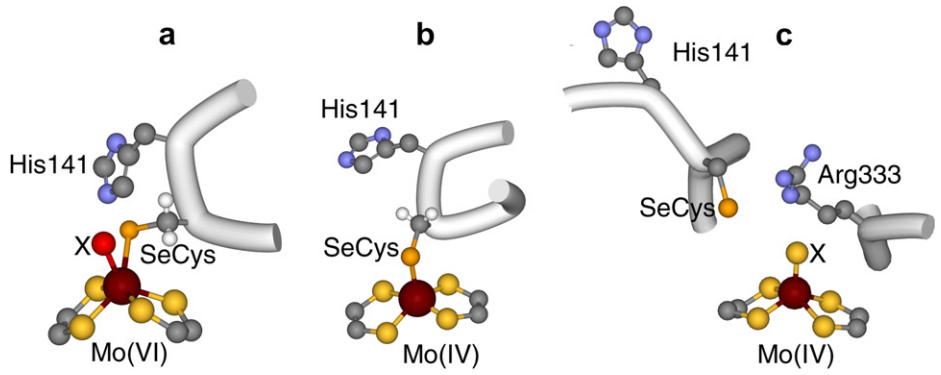

Fig. 1. Coordination around molybdenum in *Ec* Fdh-H (adapted from Ref. [1]) (a) oxidized form. (b) Formate reduced form as determined in Ref. [1]. (c) Idem (b) but as determined in Ref. [10].

in *Ec* Fdh-N [3], *Ec* Fdh-H [1,8], and *Dd* Fdh [9], but X-ray data obtained for *Dg* Fdh suggest the presence of a sulfur species [5,6]. The latter is in line with a recent reevaluation of the crystallographic data of *Ec* Fdh-H reduced by formate, on the basis of which it is proposed that ligand is better fitted as a sulfur species (=S or –SH) than a oxygenic species (Fig. 1c) [10]. In contrast to the hexacoordinated Mo site observed in the oxidized enzyme, X-ray data of the formate reduced form of *Ec* Fdh-H shows a Mo site in distorted square-pyramidal coordination, in which the four sulfur atoms from two pterin moieties occupy the base of the pyramid. In the original crystal structure, the ligand situated in the apical position was identified as the selenium atom from a selenocysteine residue (Fig. 1b) [1], but the recent reinterpretation of the crystal data demonstrated that ligand X occupies this position (Fig. 1c) [10]. It is important to note that all the crystallographic studies in *Ec* Fdh-H were performed with enzymes samples purified in the presence of azide, which is not the case for *Dd* Fdh. This remark will be useful in the analysis of our results.

The postulated reaction mechanism for formate dehydrogenases implies the binding of the formate molecule to Mo(VI) ion followed by the concomitant CO_2 release and reduction to Mo(IV). Then, Mo(IV) is converted to Mo(VI) through a two electron sequential transfer to an external electron acceptor [1,11]. This process occurs via cleavage of the C–H bond to release CO_2, indicating that, in contrast to most hydroxylases, formate oxidation does not incorporate oxygen from solvent [11,12].

EPR studies performed on formate reduced *Ec* Fdh-H , which must be purified in the presence of azide to prevent enzyme inactivation, gives a nearly axial EPR signal having a hyperfine coupling with the α-proton from formate. This EPR-active species, called *2.094*, was associated with a Mo(V) intermediate during the reoxidation from Mo(IV) to Mo(VI) when catalysis occurs [12]. *Dd* Fdh, which does not need azide to prevent enzyme inactivation, develops a distinct Mo(V) EPR signal upon formate reduction in absence of azide [13]. This signal (hereafter called *formate* signal) shows also hyperfine structure, apparently with nuclear species with $I = 1/2$.

In this work, we report detailed EPR studies of the Mo(V) forms obtained on formate reduction of both as-prepared and inhibited formate dehydrogenase from *Desulfovibrio desulfuricans* ATCC 27774. The work is oriented to elucidate the nature of the ligands to Mo giving the hyperfine structure of the *formate* signal and how the Mo(V) site of the formate reduced enzyme is modified upon inhibition. The relationship between the *2.094* species detected in *Ec* Fdh-H and the *formate* species is also discussed.

2. Materials and methods

2.1. Cell growth and protein purification

Dd Fdh was isolated as reported before [13] but with some modifications. Cells (800 g wet weight) were suspended in 10 mM Tris–HCl buffer and ruptured in a French press at 9000 psi. After centrifugation (10,000×g, 45 min) and ultracentrifugation (180,000×*g*, 60 min) the supernatant was dialyzed against 10 mM Tris–HCl buffer. The soluble extract was subjected to a four step purification protocol that included: two anionic exchange chromatographies (DEAE–Cellulose™ and Q-Sepharose™ columns equilibrated with 10 mM Tris–HCl and eluted with a linear gradient to 250 mM Tris–HCl), a Hydroxyapatite column (equilibrated with 100 mM Tris–HCl and eluted with a potassium phosphate linear gradient from 1 to 200 mM), and a Superdex 200 (Pharmacia) column (equilibrated with 300 mM Tris–HCl). In contrast to what has been previously observed [13], no loss of activity was detected after the Hydroxyapatite column. All purification procedures were performed under aerobic conditions at 4 °C and pH 7.6. This procedure yielded approximately 30 mg of active enzyme with a specific activity of 143(6) μmol/min/mg. *Dd* Fdh, like other formate dehydrogenases from sulfate reducing bacteria [14,15], can be purified under aerobic conditions without loss of activity. Protein quantification was performed using both the Lowry method and the bicinchoninic acid kit from Sigma using bovine serum albumin as standard protein. Molybdenum, iron and selenium

were quantified by induced coupled plasma emission spectroscopy. Metal content was similar to that reported in previous works [9,13].

2.2. Activity assays

Enzymatic assays were performed under strict anaerobic conditions (small amounts of oxygen inhibit the reaction) following the reduction of benzyl viologen at 555 nm as previously described [13] but with some modifications. Enzyme (35 nm) was pre-incubated for 10 min at 37 °C with a solution containing 130 mM β-mercaptoethanol in 60 mM Tris–HCl, pH 7.6. Then, different amounts of sodium formate were added and incubated for 10 min. The reaction was started by the addition of 7.5 mM benzyl viologen. The addition order of the reagents constitutes the main difference with the previously reported [13]. Kinetic parameters were calculated assuming a Michaelis–Menten model. Similar results were obtained by direct linear plot and by fitting the raw data. Inhibiting effects were tested adding azide or cyanide to the reaction mixture. Inhibition type was determined using the Dixon and direct linear plot as described in Ref. [16]. The values of K_{ic} and K_{iu} (competitive and uncompetitive inhibition constants, respectively) were calculated by varying the concentration of substrate and inhibitor as described in [16].

2.3. EPR spectroscopy

X-Band spectra were recorded on a Bruker EMX spectrometer equipped with a dual-mode cavity (Model ER4116DM) and an Oxford Instrument continuous flow cryostat. Simulations were performed with the program Q-powa [17]. The spectra were acquired in non-saturating conditions Experimental conditions: Microwave frequency, 9.65 GHz; Modulation frequency, 100 kHz, Modulation amplitude, 5G; Microwave power, 2 mW; Temperature, 100 K.

Samples for EPR spectroscopy were prepared in 60 mM Tris–HCl buffer, pH 7.6. Mo(V) EPR signals were developed by reducing as-prepared or D_2O exchanged *Dd* Fdh samples (200 μM) with formate (HCOONa or DCOONa, D. 99% from Cambridge Isotopes) using a molar ratio formate:enzyme of 30:1 in three different conditions, as explained in results. Samples were frozen immediately in liquid nitrogen after addition of reductant followed by spectral acquisition. Then, they were thawed and incubated for 5 min, after which they were immediately frozen for spectral acquisition. This procedure was repeated 6 times. Longer incubation times showed no significant changes in the spectra. All the procedures were performed under strict anaerobic conditions. As-prepared *Dd* Fdh samples present a rhombic EPR signal [13] which keeps line-shape and intensity on reduction either with formate or dithionite and was subtracted in all the spectra.

Inhibited samples were prepared by dialyzing *Dd* Fdh (200 μM) against H_2O–buffer or D_2O–buffer (D. 99.9% from Cambridge Isotopes) containing inhibitors (cyanide or azide) with a final concentration of 3 mM and then reduced with dithionite (molar ratio dithionite: enzyme, 200:1) or formate, as explained to obtain the *formate* signal (see Section 3). For the formate or dithionite reduced samples, the inhibitors were added in anaerobic conditions to reach a concentration of 3 mM.

3. Results

3.1. Kinetic properties of Dd Fdh

As previously published [13], the steady-state analysis shows a Michaelian behavior with a similar K_m [65(8) μM], but with the turnover number (k_{cat}) remarkably increased from 12 s^{-1} to 357(18) s^{-1}. The main difference from the procedures reported in Ref. [13] is that, in the present work, the reaction is started with the enzyme previously reduced by formate. This result suggests that the enzyme ready state is achieved when the as-prepared sample is pre-reduced with the substrate. Similar behavior was observed in other molybdoproteins [18]. The effect of azide and cyanide as inhibitors was tested. Both inhibitors showed a mixed-inhibition pattern with $K_{iu} = 214(3)$ μM and $K_{ic} = 33(3)$ μM for azide, and $K_{iu} = 1634(156)$ μM and $K_{ic} = 420(127)$ μM for cyanide.

3.2. EPR of Formate-reduced enzyme

Fig. 2a shows the *formate* signal obtained by reduction of as-prepared *Dd* Fdh after 30 min incubation with $HCOO^-$. The line-shape of the signal at shorter incubation times is similar to that of Fig. 2a but with lower intensity. The spectrum of Fig. 2a is rhombic and shows hyperfine structure from nuclear species with $I = 1/2$. To determine the nature of these splittings, this signal was obtained in different experimental conditions explained below.

Firstly, to confirm whether the hyperfine structure is given by the formate α-proton, the as-prepared enzyme was reduced with $DCOO^-$. This experiment reproduces the results obtained for the as-prepared enzyme (see Fig. 2a) confirming that, under these experimental conditions, splitting from formate α-proton can be neither detected nor solved.

Secondly, to determine whether the hyperfine splittings are given by solvent exchangeable protons, the *formate* signal was developed with the enzyme exchanged into D_2O-buffer using $HCOO^-$ as reductant. Again, from circa 5 s to 30 min of incubation time the intensity of the signal increases but the line-shape does not change. The spectrum obtained after 30 min incubation is shown in Fig. 2b. In conclusion, comparison of Fig. 2a and b clearly indicates that the *formate* signal is split by two types of $I = 1/2$ nuclei (g- and A-values in caption to Fig. 2). One of the nuclear species is not solvent exchangeable and splits the g_{max} feature but not the two lowest g-values (Fig. 2b). According to the proposed structure for the oxidized active site of *Dd*

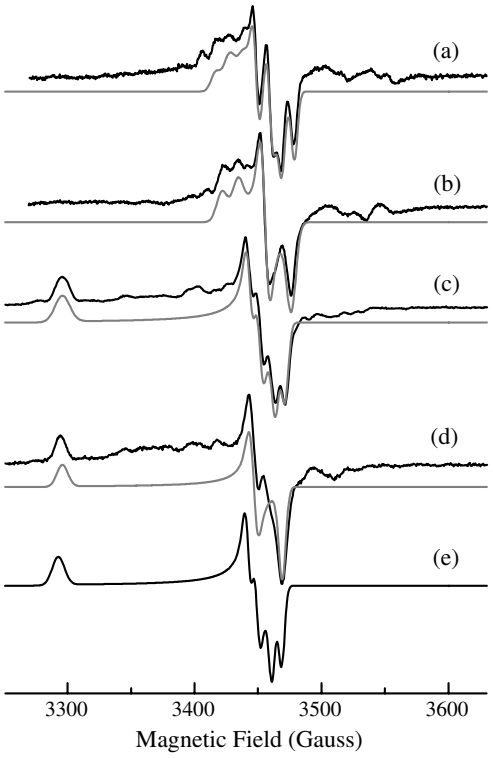

Fig. 2. Mo(V) EPR signals at 100 K of *Dd* Fdh together with simulation. (a) as-prepared enzyme after 30 min reduction with $HCOO^-$. (b) Idem (a) but with the enzyme exchanged into D_2O-buffer. (c) Azide inhibited enzyme reduced with $HCOO^-$ for ~5 s, and (d) idem (c) but reduced with $DCOO^-$. (e) EPR simulation of the *2.094* signal obtained in *Ec* Fdh-H. EPR parameters: (a) $g_{max} = 2.012$ (12), $g_{mid} = 1.996$ (8), $g_{min} = 1.985$ (8), $A^1_{max} = 11.7$, $A^2_{max} = 7.7$, $A^2_{mid} = 10.0$, $A^2_{min} = 9.3$; (b) $g_{max} = 2.012$ (12), $g_{mid} = 1.996$ (8), $g_{min} = 1.984$ (12), $A^1_{max} = 11.7$. (c) $g_{max} = 2.092$ (20), $g_{mid} = 2.000$ (9), $g_{min} = 1.989$ (9) $A_{max} =$ n.d., $A_{mid} = A_{min} = 7.0$, (d) $g_{max} = 2.092$ (15), $g_{mid} = 2.000$ (10), $_{min} = 1.988$ (9) (e) $g_{max} = 2.094$, $g_{mid} = 2.000$, $g_{min} = 1.989$, $A_{max} = 2.5$, $A_{mid} = 6.3$, $A_{min} = 7.0$ (taken from [11]). Linewidths in MHz are given in parentheses. A in $cm^{-1} \times 10^{-4}$. Indexes 1 and 2 stand for the non-solvent and solvent exchangeable protons, respectively.

Fdh [9], the closest non-solvent exchangeable protons to the Mo atom are situated on the β-methylene carbon of the selenocysteine ligand, which suggests that this ligand is also coordinated to the Mo(V) ion. Anisotropic splittings associated with the hydrogen atoms of β-methylene carbon of the selenocysteine (or cysteine) have been observed in several members of the DMSO reductase family of enzymes [19]. The second splitting is produced by species exchangeable with solvent and is detectable at the three principal *g*-values. The magnitude of this interaction, which is more isotropic, suggests that it should be produced by protons of a Mo-ligand situated in the first coordination sphere, and hence they should be associated with the ligand X present in the structure of the oxidized active site (Fig. 1a).

3.3. EPR of inhibited enzyme

Since azide and cyanide are inhibitors of *Dd* Fdh, we investigated their effect in two different experimental setups. One of them aimed to detect the Mo(V) species obtained in inhibited enzyme samples followed by formate or dithionite reduction. The second experiment pursued to address the effect of both inhibitors on the *formate* species.

For the first experiment, as-prepared *Dd* Fdh inhibited by azide was reduced with $HCOO^-$ and $DCOO^-$, respectively. The same procedure was carried out in D_2O exchanged samples. The spectra of the as-prepared *Dd* Fdh reduced by $HCOO^-$ and $DCOO^-$ and immediately frozen are shown in Fig. 2c and d, respectively. In contrast to that observed for the formate signal, the intensity of the spectra, does not change along time. These spectra show nearly axial symmetry with resonance lines split at g_{med} and g_{min} when using $HCOO^-$ as reductant (EPR parameters in caption to Fig. 2). This splitting is not detected in the sample reduced with $DCOO^-$, which confirms that $I = 1/2$ species interacting with the Mo(V) center results from the α-proton from formate that is transferred, upon oxidation of the substrate, presumably to ligand X. Furthermore, this signal correspond to the *2.094* signal obtained in *Ec* Fdh-H on dithionite or formate reduction. A simulation of the *2.094* signal in *Ec* Fdh-H is shown in Fig. 2e for comparison. Similar results were obtained using cyanide instead of azide.

An interesting feature of the Mo(V) signal obtained in the presence of azide is that the splitting produced by the formate α-proton is exchangeable with solvent along time. This is shown in Fig. 3, which only includes the g_{med} and g_{min} features of the *2.094* signals obtained at two different incubation times and in two conditions (see caption). The splitting of the D_2O-exchanged enzyme reduced with $HCOO^-$ (black line in Fig. 3a) disappears after 15 min incubation by the replacement of the formate α-proton atoms for D atoms (gray line in Fig. 3a). A similar process,

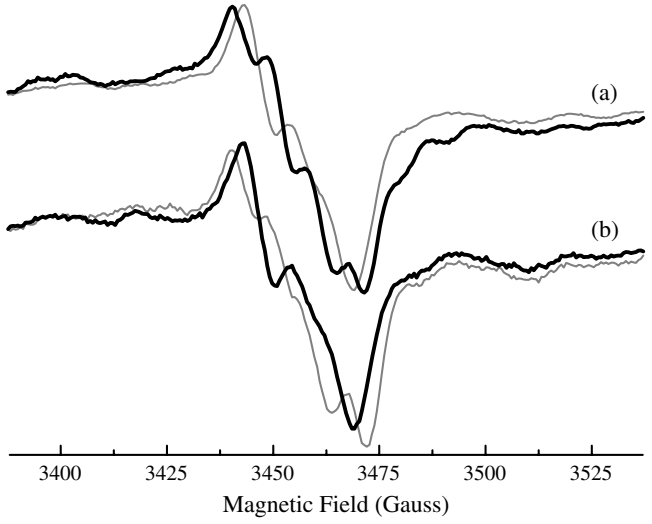

Fig. 3. Mo(V) EPR signals of azide inhibited *Dd* Fdh. (a) Enzyme exchanged into D_2O-buffer reduced with $HCOO^-$, and (b) as-prepared enzyme reduced with $DCOO^-$. Spectra given in black and gray lines correspond to approximately 5 s and 15 min of incubation time, respectively.

but in an opposite direction, occurs with the as-prepared enzyme reduced with $DCOO^-$. In this case, no splitting is detected after immediate addition of reductant (black line in Fig. 3b), and then the D atoms from formate are changed to H from solvent with the concomitant developing of the splitting (gray line in Fig. 3b). This confirms that, under inhibiting conditions, the splitting observed in the Mo(V) signal is solvent exchangeable and originated from the formate α-proton.

The 2.094 signal can be also obtained on dithionite reduction of azide or cyanide inhibited samples of *Dd* Fdh (not shown). This indicates that the detection of this signal does not depend on the reducing agent but only on the inhibitor presence in the medium.

For the second experiment, azide or cyanide were added to samples showing the *formate* signal. The *2.094* signal was also detected under these conditions with no signs of the *formate* signal, even in the sample obtained immediately after inhibitor addition (not shown).

4. Discussion

Dd Fdh is a trimeric enzyme [13] having a catalytic subunit closely related to the monomeric *Ec* Fdh-H [7]. These similar structural properties are reflected in the kinetic behavior towards substrate and inhibitors. Both enzymes follow a Michaelian-type kinetic and are inhibited by azide. Azide is a mixed inhibitor with respect to formate in both *Ec* Fdh-H ($K_i = 75$ μM) and *Dd* Fdh [$K_{iu} = 214(3)$ μM and $K_{ic} = 33(3)$ μM]. Cyanide also displays a mixed inhibition pattern with respect to formate with $K_{iu} = 1634(156)$ μM and $K_{ic} = 420(127)$ μM. There is no report for cyanide inhibition in *Ec* Fdh-H.

In contrast to any unspecific reductant (e.g., dithionite), formate, the natural substrate of the enzyme, is a specific reductant of Fdhs. For example, formate cannot reduce benzyl viologen like dithionite, and also cannot reduce Mo-containing enzymes of the same family, e.g., nitrate reductases. It means that the reduction of the enzyme by formate must be driven by a specific reaction and its reducing effect must necessarily be accomplished through an interaction between the formate and the active site. The electrons given to the enzyme by formate are distributed along the electron transfer pathway according to their relative values of redox potentials. Furthermore, as discussed below, both Mo(V) species (*formate* and 2.094) do not correspond to substrate-bound forms, and hence they should correspond to Mo(V) species obtained after CO_2 release.

The distinct symmetries of the *formate* and *2.094* EPR signals in *Dd* Fdh suggest Mo(V) sites with different coordination geometries. The hyperfine splittings detected in the *formate* signal (Fig. 2a and b) indicate that the selenocysteine residue and the ligand X (hydroxyl or sulfydryl) are coordinated to molybdenum. Our current interpretation for the structure of the *formate* species is shown in Fig. 4a. The coordination around the Mo(V) ion is similar to that proposed by EXAFS for the Mo(VI) ion site of *Dd* Fdh [9] and the X-ray structures of the oxidized forms of *Ec* Fdh-H [1], *Ec* Fdh-N [3], and *Dg* Fdh [6] (Fig. 1a shows the structure of *Ec* Fdh-H). *Formate* species could also correspond to a Mo(V)–substrate complex. However, this possibility is in principle excluded since, in such case, formate should occupy a seventh coordination position, which is unlikely for a member of the DMSO family [7].

Mo(V) EPR signals having nearly axial symmetry, such as the one observed for the *2.094* signal, are usually given by Mo sites in approximately square pyramidal coordination, as those found in the proteins of the xanthine oxidase family of enzymes [20]. The detection of a hyperfine coupling in the *2.094* signal associated with a proton of a molybdenum ligand, together with the recent reinterpretation of the crystal data of *Ec* Fdh-H [10], lead to the tentative proposal for the structure of the *2.094* species shown in Fig. 4b, in which ligand X (hydroxyl or sulfidryl) occupies an apical position. The possibility that the *2.094* species could correspond to a formate-bound form is excluded as the *2.094* signal can be also obtained using dithionite as reductant [12]. Following the same reasoning, the two inhibitor molecules (azide and cyanide) yield the same EPR signal, also suggesting that none of them should be bound to the molybdenum active site. This is in contrast with kinetic studies of *Ec* Fdh, in which is proposed that inhibition occurs by binding of the azide molecule to the active site [21]. Additional work is necessary to solve these discrepancies.

Our EPR data strongly support that Mo(V) pentacoordinated species are only formed in the presence of inhibitors. In this sense, it is important to remark again that *Ec* Fdh-H needs to be purified in the presence of azide to prevent inactivation by oxygen [16,17]. In the catalytic mechanism proposed on the basis of structural and EPR data obtained in *Ec* Fdh-H, the *2.094* species has been suggested as the Mo(V) intermediate in the reoxidation from Mo(IV) to Mo(VI) [10,12]. Taking into account the new information now collected for *Dd* Fdh, the *2.094* species are intermediates in the reoxidation of molybdenum when azide is present.

The re-evaluation of the three-dimensional structure of the formate reduced *Ec* Fdh-H in the presence of azide shows that the SeCysteinyl ligand is not coordinated to Mo(V). Based on this observation the author suggests a completely different enzymatic mechanism for this group of enzymes [10]. However, this proposal must be taken with caution since we could now demonstrate that the structure

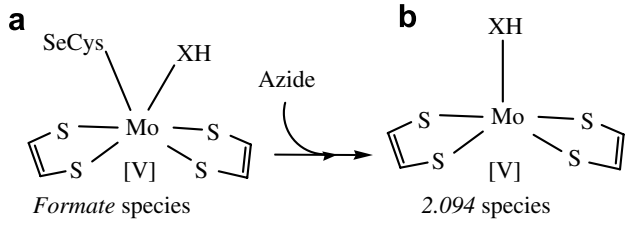

Fig. 4. (a) Postulated coordination around molybdenum for the *formate* species. (b) idem (a) but for the *2.094* species.

of the Mo active site depends on the enzyme preparation and consequently different species could be involved in the reaction mechanism of formate oxidation in absence of inhibitors. Work focused on the clarification of mechanistic aspects operating in this family of enzymes is ongoing.

5. Abbreviations

Fdh	formate dehydrogenase
Ec	*Echerichia coli*
Dg	*Desulfovibrio gigas*
MGD	molybdopterin guanine dinucleotide
Dd	*Desulfovibrio desulfuricans* ATCC 27774
EXAFS	extended X-ray absorption fine structure

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