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The Formate Dehydrogenase Isolated from the Aerobe *Methylobacterium* sp. RXM Is a Molybdenum-Containing Protein

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The formate dehydrogenase (FDH) isolated from cells of *Methylobacterium* sp. RXM grown on molybdenum-containing mineral medium using methanol as carbon source, was partially purified (at least 90% pure as revealed by SDS-PAGE). The enzyme is unstable under oxygen and all the purification steps were conducted under strict anaerobic conditions. The molecular mass is 75 kDa (gel exclusion 300 kDa). The enzyme was characterized in terms of the kinetic parameters towards different substrates and electron acceptors, pH and temperature dependence and the effect of a wide range of compounds in the enzymatic activity. The EPR spectra of the dithionite reduced sample show, at low temperature (below 20 K), two rhombic EPR signals due to two distinct [Fe-S] centres (centre I at g-values 2.023, 1.951 and 1.933, and centre II at g-values 2.054 and 1.913). At high temperature (around 100 K) another rhombic EPR signal is optimally observed at g-values 2.002, 1.987 and 1.959 and attributed to the molybdenum site. The EPR signals assigned to the iron-sulfur centres show a strong analogy with the aldehyde oxido-reductase from *Desulfovibrio gigas* known to contain a Mo-pterin and two [2Fe-2S] centres and whose crystallographic structure was recently resolved. © 1997 Academic Press

When pink-pigmented facultative methylotrophs are grown on methanol, the last step of the linear dissimilatory pathway for reducing equivalents generation is catalysed by a NAD^+-dependent formate dehydrogenase (FDH E.C.1.2.1.2). This enzyme has an important regulatory role being under the control of the adenine nucleotide pool (3).

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Amongst aerobic bacteria and yeasts, NAD^+-dependent formate dehydrogenases have been purified and characterized from *Moraxella* sp. C-1 (4), *Pseudomonas* sp. 101 (former *Achromobacter parvulus*) (5,6), *Candida methanolica* (7,8), *Pichia pastoris* (9) and *Candida methylica* (10). All of them have similar molecular properties being a homogeneous family showing two identical sub-units, low molecular mass (around 90 kDa), low affinity for formate and been indicated to not contain any group prosthetic or metal ions (3,11).

However, there are exceptions. For example, the enzymes from *Pseudomonas oxalaticus* and *Alcaligenes eutrophus*. The first is a high-molecular mass complex flavoprotein (315 kDa), containing two flavin mononucleotide molecules, non-heme iron and acid-labile sulfur atoms (12) and the other is a heterotetramer of 197.000 kDa containing one flavin mononucleotide and iron-sulfur centers (13). In addition, physiological evidence suggested that in presence of molybdenum, aerobic FDHs from *Pseudomonas* sp. 101 (14), *Mycobacterium vaccae* 10 (15) and *Methylobacterium* sp. RXM (16,17) could resemble the molecular properties of FDHs isolated from anaerobic organisms which are characterised to be complex iron-sulfur-containing proteins, some containing also molybdenum or tungsten (3, 11, 18, 19). Recently, a formate dehydrogenase was isolated and crystallized from *Escherichia coli,* a facultative anaerobe, containing selenium in the molybdenum coordination sphere (20).

This paper shows the first spectroscopic evidence demonstrating that the aerobic formate dehydrogenase from *Methylobacterium* sp. RXM is a molybdo-protein containing iron-sulfur centres.

MATERIALS AND METHODS

Growth of organism. *Methylobacterium* sp. strain RXM is a serine-type pink-pigmented facultative methylotroph (PPFM) and was

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TABLE I

Purification Steps of the Formate Dehydrogenase from Aerobe *Methylobacterium* sp. RXM

Purification step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Cytoplasmatic fraction	170	333.0	909.5	0.37	(100)
Q-Sepharose	80	123.5	260.9	0.47	37.1
Q-Sepharose	37	60.0	42.1	1.74	18.0
DEAE MemSep 1000	13	6.6	3.6	1.80	2.0

isolated and maintained as described earlier by Gírio & Attwood (1991).

The bacterium was grown in mineral medium (17) and filter-sterilized methanol (100 mM) was added as carbon source. Several 2 liter erlenmeyer flasks were filled with 500 ml of mineral medium and inoculated with 5% (v/v) of 24h-old shake flask cells. The culture incubations were carried out on an orbital-shaker at 30°C at 150 rpm. These cultures were used to inoculate a 50 litres pilot fermenter (Biolafitte, St. Germain-en-Lay, France) with 30 litres of working volume. Late-exponential cells (OD ~ 4.0) were harvested with a Westfalia continuous-flow centrifuge, at a cell concentration of 4.0 g (wet mass)/liter. The obtained biomass was then washed with cold 50 mM Tris-HCl buffer (pH 7.5) and ressuspended in the same buffer (1/4, w/v) containing 1 mM phenylmethylsulfonyl fluoride (PMFS) for sonication at 80 W for 10 min at 4°C. Cell debris was removed by high-speed centrifugation (27,000 *g*, 30 min, 4°C) and the supernatant was used as cell-free extract for enzyme purification assays.

Formate dehydrogenase assay. Formate dehydrogenase (E.C.1.2.1.2) was routine spectrophotometrically assayed as described before (17) using NAD^+ as electron acceptor. For all enzymatic assays a linear relationship between reaction rate and enzyme concentration was found. Enzyme units (U) were defined as the amount in μmol of NADH oxidized or NAD^+ reduced per min at the assay conditions. Specific activity was expressed as U/mg of protein.

Protein determination. Soluble protein was assayed as described in (21). Bovine serum albumin and globulin was used as standard protein.

Electrophoresis and molecular mass determination. Purity of the formate dehydrogenase was established by polyacrylamide gel electrophoresis and molecular mass and subunit structure were determined by SDS acrylamide gel electrophoresis using the Pharmacia low molecular mass kit as standard.

The molecular mass of the purified protein was also estimated by gel filtration using a Superdex 200HR 10/30 column (Pharmacia).

Spectroscopic methods. UV/visible optical spectra were recorded on a Shimadzu UV-2101PC split-beam spectrophotometer using 1 cm quartz cells. EPR measurements were performed on a Bruker ER 200 tt spectrometer equipped with an Oxford Instruments continuous helium flow cryostat.

Enzyme purification. All purification procedures were performed at 4°C and pH 7.6 under strict anaerobic conditions. The cytoplasmic fraction was obtained after centrifugation at 25,000 $\times g$ for 30 min in a Beckman L-70 Ultracentrifuge (170 ml) and then it was loaded into a Q-sepharose column (18 $\times$ 1.6 cm) equilibrated with 10 mM Tris/HCl buffer. All buffers containing sodium dithionite (2 mM), dithiothreitol (5 mM), and glycerol (10%, v/v). A Tris/HCl gradient (10 - 700 mM) was applied with a total volume of 2 l. The fraction containing mostly all formate dehydrogenase activity was eluted around 500 mM. It was diluted 1:2 with 10 mM Tris/HCl and applied in the same column and eluted at same ionic strength. The formate dehydrogenase fraction was concentrated on a Diaflo apparatus with a YM30 membrane and loaded in a small DEAE column MemSep 1000 (Millipore). The specific activity after this purification step was 1.8 U/mg of protein (Table I).

Kinetic measurements, pH, and temperature dependence of the enzymatic activity and inhibitor studies. Kinetic parameters were extracted following enzymatic activities determined as previously described. Enzyme concentration was fixed and different electron acceptors and substrates tested. K_m and V_{max} values varying NAD^+ and formate concentrations. pH dependence of the enzymatic activity was measured at 50 mM ionic strength in a pH range from 5.5 to 10, using 2-[N-Morpholino] ethane-sulfonic acid (MES), N-[2-Hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid] (HEPES) and Tris/HCl buffer systems. For temperature dependence studies, the enzymatic activities were measured after 10 min incubation of the protein at a given temperature. The effect of a wide range of chemical compounds was studied on the enzymatic activity. Concentration and incubation time in the presence of different inhibitors was varied in order to infer on their action, as indicated in the Results Section.

RESULTS

The formate dehydrogenase isolated from *Methylobacterium* sp. RXM (Table I) was analysed by SDS/PAGE electrophoresis. A major band was stained for protein at 75 kDa, that accounts for more than 90% of the total gel coloration. Under non-denaturating conditions the protein behaves as a tetrameric complex.The enzyme has an optimal activity at pH 7.5 - Tris/HCl buffer and displays an alkalotolerant activity (at pH 10.0 exhibits 57% of the maximal activity). The effect of temperature on the stability of the enzyme was also carried out. No loss of activity was observed up to 35°C. Between 35°C and 65°C a decrease of activity occurred and 100% loss of the initial activity was observed at 65°C for 10 min. The enzyme was stable on incubation at room temperature for 10 min in a wide pH range, 6.5-10.0.

The ability of the enzyme to catalyse the oxidation of the following compounds was studied under the standard assay conditions: formaldehyde, methanol, succinate, ethanol, acetate, citrate and pyruvate. The enzyme showed a high substrate specificity for formate, being the only compound where oxidative activity was detected. The enzyme is fully specific to NAD^+ do not exhibiting activity towards $NADP^+$. However, it accepts artificial electron acceptors, such as potassium ferricyanide.

Kinetic studies on formate oxidation were carried out. The activities of formate dehydrogenase were measured at various concentrations of formate (by keeping constant NAD^+ concentration). The enzyme follows a

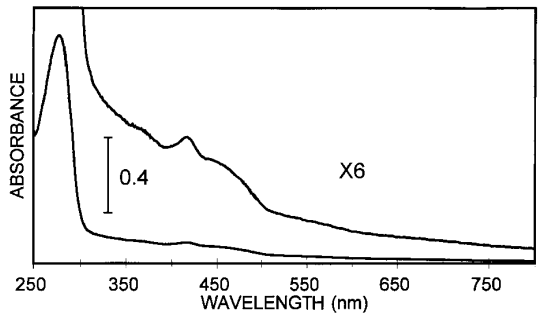

FIG. 1. UV/visible absorption spectrum of the *Methylobacterium* sp. RXM FDH.

Michaelis-Menten kinetic and the K_m and V_{max} values were obtained from Hanes plots - K_m values for formate 4.7 mM and for NAD^+ 2.6 mM. The inhibitory effects of several compounds on the enzyme activity were investigated. High sensitivity of formate dehydrogenase from *Methylobacterium* sp. RXM was observed to *p*-chloromercuribenzoate, imidazole, $CuCl_2$ and $ZnCl_2$ (78%, 32%, 72% and 95% of inhibition, respectively). The addition of β-mercaptoethanol to the assay after the inhibition with *p*-chloromercuribenzoate restored the initial enzyme activity (>90%). In addition it was observed an enhanced enzyme activity after the addition of dithiothreitol, a thiol-protecting agent. Azide was found to be inhibitor even at very low concentration (0.1 mM) which apparently correlates with the presence of molybdenum associated to the enzyme active site. The sensitivity to NADH and ATP is in agreement with the formate dehydrogenase *in vivo* physiological role of catalysis of a energy-generating reaction as source of reducing power in the methanol metabolism by *Methylobacterium* sp. RXM.

The UV/Visible absorption spectrum of formate dehydrogenase is presented in Figure 1. The protein exhibits an absorption peak at 280 nm and broad charge transfer bands in the 400 nm spectral region. The overall spectrum is dominated by the charge transfer bands due to the [Fe-S] centers. This spectrum reminds those observed for the deflavo-forms of xanthine and *D. gigas* aldehyde oxido-reductase (1). A minor contamination of a cytochrome component is observed better seen at the Soret band region.

The *Methylobacterium* sp. RXM formate dehydrogenase sample is almost EPR silent as isolated. After 15 min reduction with dithionite under anaerobic conditions, a complex signal develops. Figure 2 shows a set of EPR spectra obtained in a wide range of temperature (10-100 K). Rhombic EPR signals developped in the reduced samples, assigned to two distinct iron-sulfur centers with g-values at 2.023, 1.951 and 1.933 (center I) and 2.054 and 1.913 (center II). Center II shows fast relaxation than center I and is to broad to be detected above 25 K. At low temperature, the weak central features reveal the presence of a EPR signal with gav < 2 are assigned to Mo(V) species. At high temperature (around 100 K), the iron-sulfur centers are not detectable and the Mo(V) EPR signal is better visualized. The signal has a rhombic shape and was simulate with g-values 2.002, 1.987 and 1.959 as shown in Figure 2, Panel B. The Mo(V) signal observed shows analogies with one of the Mo(V) species detected in the formate dehydrogenase isolate from *P. aeruginosa* generated in the presence of low concentrations of formate with g-values at 2.012, 1.985 and 1.968 (22). The EPR signals due to the iron-sulfur centers are reminescent of the ones detected for the aldehyde oxido-reductase isolated from *Desulfovibrio gigas* known to contain a Mo-pterin site and two [2Fe-2S] centers (2, 23) (see Table II).

DISCUSSION

Formate dehydrogenases (EC 1.2.1.2) carry out an important biological reaction: oxidation/reduction of the couple formate/CO_2. Formate is a chemical compound widely used by anaerobic and aerobic orgasnims, including eukaryontes (yeast and plants), eubacteria and archaebacteria (11). Formate is produced and consumed in a variety of metabolic pathways and appears also as an important product involved in the last steps of degradation of organic matter. Formate dehydroge-

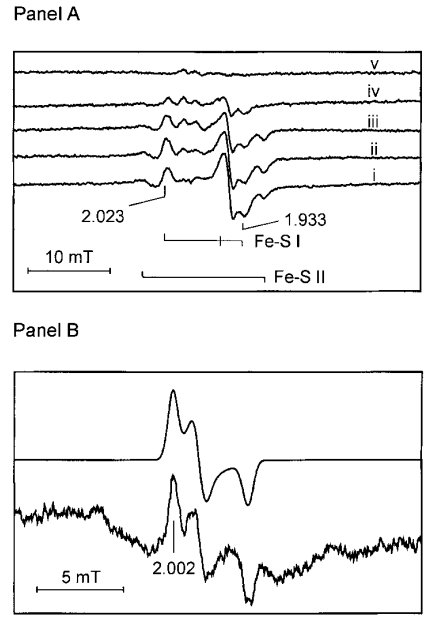

FIG. 2. EPR spectra of *Methylobacterium* sp. RXM FDH. Panel A - X-Band EPR spectra of the FDH dithionite reduced. Temperature dependence of the spectra i)10K, ii)15K, iii)25K, iv)45K and v)100K. Panel B - Detail of the X-band EPR spectrum of Mo(V) species at 100K. A simulation of the spectral data is included (g-values (linewidth, mT):2.002 (1.14), 1.987 (1.32), 1.959 (1.22)). Experimental conditions: Modulation Amplitude, 1.0 mT; Microwave frequency, 9.49 GHz; Microwave Power, 2.35 mW.

TABLE II

EPR Parameters for the Fe-S Centers of the FDH Isolated from the Aerobe *Methylobacterium* sp. RXM: Comparison with Data from the Literature

	g_1	g_2	g_3
Fe/S center I			
RXM	2.023	1.951	1.933
D. gigas AOR	2.021	1.938	1.919
Eukaryontes	2.121 ± 0.004	1.933 ± 0.005	1.907 ± 0.01
Fe/S center II			
RXM	2.054	n.d.	1.913
D. gigas AOR	2.057	1.979	1.900
Eukaryontes	2.103 ± 0.005	2.002 ± 0.006	1.912 ± 0.01

Ref. 1.

nase has also been recognized as an industrially important enzyme, particularly due to its use for the production of optically active compounds (8). Other potential industrial applications include formate determination using enzymatic kits (12) and hydrogen production from organic fuels (9).

The characterization of bacterial formate dehydrogenases, included in the wide enzyme group of the molybdenum (tungsten) hydroxylases, has given an important contribution to the participation of these rare metal components in biological systems and also to the role of selenium (24, 25). In fact, these metals have long time ago been identified as components of formate dehydrogenases and other enzymes, although only for anaerobic organisms. The participation of tungsten in related systems is less explored. However, tungsten is a topic of renewed interest and was long time ago recognized as an important component in some formate dehydrogenases from anaerobic bacteria (18, 19, 25). It was also shown to be present in some methanogens and hyperthermophiles derived enzymes.

The diverse biological reactions catalysed by formate dehydrogenases reflect the type and number of electron acceptors utilized, as well as in the different physiological roles performed, celular localization, specificity towards substrates, physiological acceptors used and content in prosthetic groups. Formate utilization is then carried out by an heterogeneous group of enzymes which involve or not metal centers (selenium, molybdenum, tungsten, non-heme and heme-iron) use or not pyridine nucleotides (11).

This heterogeneous group of enzymes can be described into two major families. The first includes a diverse group of complex iron-sulfur containing proteins, present in general in anaerobic bacterial systems. The second comprises NAD^+-dependent formate dehydrogenase, the simplest examples of pyridine nucleotide-dependent dehydrogenases. 3D structures of these enzymes, containing no metal neither cofactors, are known and the enzymes have been described as model catalytic systems for mechanistic steps involving hydride ion transfer (3) They were demonstrated to be cofactor regenerating systems and their applications in organic asymetrical synthesis of high-value-added products have been pointed out.

At present, formate dehydrogenases from aerobic organisms have been reported to be a homogeneous family containing two identical sub-units without metals or prosthetic groups (11). Recently, some of us reported that the second type of FDH could be present in an aerobic methylotrophic bacteria, *Methylobacterium* sp. RXM (17). This enzyme exhibited requirement for molybdenum to express full activity. When molybdenum is replaced by tungsten in the growth medium, a similar induced formate dehydrogenase activity was observed (16,17).

Methylobacterium sp. RXM formate dehydrogenase, now isolated and purified almost to homogeneity, has the characteristics of the enzymes so far described in anaerobic bacteria: the enzyme is a molybdenum containing enzyme, closely related to the Mo-hydroxylase family of enzymes, namely the aldehyde oxido reductases (AOR) purified from *Desulfovibrio gigas* and *D. desulfuricans* ATCC 27774, sulfate reducing bacteria (1), but no aldehyde oxido-reductase activity (using DCPIP as electron acceptor) could be detected. EPR g-values of the [Fe-S] centers and the relaxation behavior are reminiscent of the properties of molybdenum hydroxylases (Table II). In the future, we will focus on the purification of the enzyme present in the tungsten grown media, in order to verify if the same enzyme can substitute molybdenum by tungsten in the formate oxidation to CO_2.

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