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Formylmethanofuran dehydrogenase from methanogenic bacteria, a molybdoenzyme

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Formylmethanofuran dehydrogenase, a key enzyme of methanogenesis, was purified 100-fold from methanol grown *Methanosarcina barkeri* to apparent homogeneity and a specific activity of 34 μmol·min^{-1}·mg protein^{-1}. Molybdenum was found to co-migrate with the enzyme activity. The molybdenum content of purified preparations was 3–4 nmol per mg protein equal to 0.6–0.8 mol molybdenum per mol enzyme of apparent molecular mass 200 kDa. Evidence is presented that also formylmethanofuran dehydrogenase from H_2/CO_2 grown *Methanobacterium thermoautotrophicum* (strain Marburg) is a molybdoenzyme.

Methanofuran; Methanogenesis; Molybdoenzyme; Coenzyme F420; (*Methanosarcina, Methanobacterium*)

1. INTRODUCTION

Methanogenic bacteria contain an enzyme activity which catalyzes the oxidation of formylmethanofuran (CHO-MFR) to CO_2 and methanofuran (MFR) with methyl viologen (MV) as artificial electron acceptor [1].

$$\text{CHO-MFR} + 2\ \text{MV} + H_2O \longrightarrow CO_2 + \text{MFR} + 2\ \text{MV}^- + 2\ H^+$$

The enzyme mediating this reaction has been designated formylmethanofuran dehydrogenase. It is involved in CO_2 reduction with H_2 to CH_4 [2,3] and in methanol disproportionation to CO_2 and CH_4 [4].

Formylmethanofuran dehydrogenase has, before now, not been purified. Indirect evidence is available indicating that the enzyme could contain molybdenum: (i) formylmethanofuran dehydrogenase is rapidly inactivated by cyanide [1], which is a property of many molybdoproteins [5]; (ii) the dehydrogenase catalyzes a reaction analogous to that mediated by xanthine dehydrogenases [6] and formate dehydrogenases [7–9], which are molybdoproteins [9]; and (iii) growth of methanogenic bacteria is dependent on molybdenum [10,11], the amounts required being relatively high indicating a catabolic function.

Direct evidence is presented here that formylmethanofuran dehydrogenase contains molybdenum.

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Abbreviations: Mops, morpholinopropane sulfonic acid; DTT, dithiothreitol; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; CHO-MFR, formylmethanofuran; MFR, methanofuran

2. MATERIALS AND METHODS

DEAE-Sephacel, DEAE Sepharose, Mono Q HR 10/10, Superose™ 6 and AH-Sepharose 4B were from Pharmacia (Freiburg, FRG). *Methanosarcina barkeri* (strain Fusaro) (DSM 804) and *Methanobacterium thermoautotrophicum* (strain Marburg) (DSM 2133) were from the Deutsche Sammlung von Mikroorganismen (Braunschweig, FRG). The bacteria were grown, harvested, stored at −20°C and cell extracts prepared as described [4,12,13]. Enzyme purification steps were performed under strictly anaerobic conditions in an anaerobic chamber (Coy, Ann Arbor, MI, USA).

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For the purification of formylmethanofuran dehydrogenase from *M. barkeri*, 40 ml cell extract was applied to a DEAE-Sephacel column (10 cm × 4 cm) equilibrated with 50 mM Mops/NaOH buffer, pH 7.0 (from here on referred to as buffer). The column was successively eluted with 80 ml buffer, 80 ml buffer containing 0.12 M NaCl, 140 ml buffer containing 0.32 M NaCl and 115 ml containing 2 M NaCl. The fractions with formylmethanofuran dehydrogenase activity (0.32 M NaCl) were pooled and diluted 1:3 with buffer. After passage through a 0.2 μm FP030/3 filter (Schleicher & Schuell, Dassel, FRG), the fraction was halved and each half separately chromatographed on a Mono Q HR 10/10 column as described in the legend to fig.2. The peak activity fractions were pooled and concentrated with Centricon-30 Microconcentrators (Amicon, Witten, FRG) and subjected to FPLC on Superose™ 6 (50 cm × 1.6 cm) previously equilibrated with buffer containing 0.1 M NaCl which was also used as eluent. The peak activity fractions were pooled and applied to an affinity column (0.8 cm × 3 cm) with AH-Sepharose 4B matrix and methanofuran as ligand. The coupling procedure with *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide was done as described in the manufacturer's instructions. The column was equilibrated with buffer containing 0.1 M NaCl and eluted with 2 ml buffer with 0.15 M NaCl, 3 ml with 0.2 M NaCl, 3 ml with 0.2 M NaCl, 3 ml with 0.25 M NaCl, 3 ml with 0.3 M NaCl, 3 ml with 0.5 M NaCl and 3 ml with 2 M NaCl. The 0.2–0.3 M NaCl fractions contained the formylmethanofuran dehydrogenase activity.

The enzyme from *Mb. thermoautotrophicum* was purified using an analogous procedure. Tris-HCl, pH 7.6, rather than Mops/NaOH, pH 7.0, was used. For details concerning FPLC on Mono Q the reader is referred to the legend of fig.4.

Formylmethanofuran dehydrogenase activity was determined as in [1]. Protein was quantitated by the method of Bradford with ovalbumin as standard using the Bio-Rad microassay [14]. Molybdenum contents of formylmethanofuran dehydrogenase fractions were determined by atomic absorption spectroscopy on a 3030 Perkin-Elmer atomic absorption spectrophotometer fitted with a HGA-600 graphite furnace assembly and an AS-60 autosampler. Samples were diluted with 0.2% nitric acid to the linear working range and 20 μl aliquots containing 100–800 pg of molybdenum were injected directly into the furnace assembly. The injected sample was dried for 60 s at 80°C and for 10 s at 120°C, charred for 20 s at 1500°C, and atomized at 2650°C for 6 s. Nonspecific background absorption was compensated for by Zeeman-effect background corrector.

Methanofuran was isolated from *Mb. thermoautotrophicum* [15] and formylmethanofuran was prepared as in [1].

3. RESULTS

3.1. *Purification of formylmethanofuran dehydrogenase from* Methanosarcina barkeri

Formylmethanofuran dehydrogenase was purified from methanol grown *M. barkeri* cells which had been stored at −20°C for several weeks. The specific activity of the enzyme in extracts prepared from these cells was 0.31 U/mg protein (table 1). It is 1.2 U/mg when freshly harvested cells are analyzed [1] indicating that activity was lost upon freezing, storage, and/or thawing of the bacteria. Purification was achieved by successive chromatography on DEAE-Sephacel, Mono Q, HR 10/10, and Superose™ 6 (table 1). The specific activity increased to 34 U/mg indicating a 100-fold purification. The activity yield was 10%. Polyacrylamide gradient gel electrophoresis of the Superose fraction revealed the presence of 2 major proteins of apparent molecular mass 200 kDa and 400 kDa, respectively, both of which had formylmethanofuran dehydrogenase activity. The additional presence of some minor protein bands without enzyme activity indicated that the enzyme preparation was not yet pure. These contaminating proteins could be removed by affinity chromatography on AH-Sepharose 4B containing methanofuran as the ligand (fig.1).

The molybdenum content of the cell extract was 0.22 nmol per mg protein. Chromatography on

Table 1

Purification of formylmethanofuran dehydrogenase from *Methanosarcina barkeri*

Fraction[a]	Activity[b] (U)	Protein (mg)	Specific activity (U/mg)	Molybdenum (nmol)	Molybdenum content (nmol/mg)	Activity per molybdenum (U/nmol)
Cell extract (40 ml)	318	1040	0.31	228	0.22	1.4
DEAE-Sephacel	350	288	1.2	13.2	0.046	26.5
Mono Q HR 10/10	164	16	10.2	12.8	0.8	12.8
Superose™ 6	31	0.9	34	3.8	4.2	8.2

[a] The purification procedure is described in section 2

[b] 1 U = 2 μmol methyl viologen reduced by formylmethanofuran per min

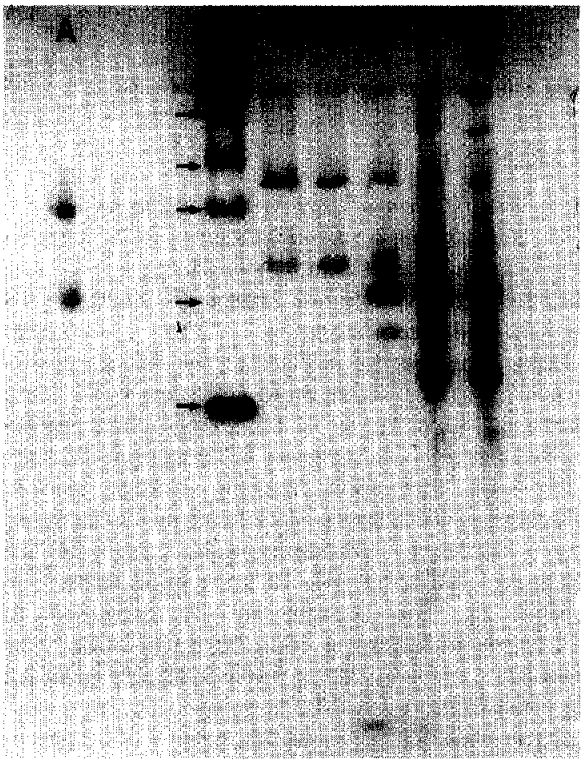

Fig.1. Polyacrylamide gel electrophoresis of formylmethanofuran dehydrogenase from *Methanosarcina barkeri*. Representative peak activity fractions from purification steps were analyzed on a 4–20% polyacrylamide gradient slab gel and stained for protein with Coomassie blue R 250 or for enzyme activity. (Lane A) Purified enzyme (21 μg) stained for activity. (Lane B) M_r standards: thyroglobulin, 670000; ferritin, 445000; catalase, 240000; aldolase, 160000; bovine serum albumin, 67000. (Lane C) Enzyme (34 μg) after FPLC on Superose™ 6. (Lane D) Enzyme (35 μg) after affinity chromatography. (Lane E) Enzyme fraction (74 μg) after FPLC on Mono Q HR 10/10. (Lane F) Enzyme fraction (87 μg) after chromatography on DEAE-Sephacel. (Lane G) Cell extract (70 μg). Lane A is displaced 4 mm with respect to lanes B–G.

DEAE-Sephacel showed that 70–90% of the molybdenum was associated with the fraction eluting at 0.12 M NaCl and that 10–30% coeluted with formylmethanofuran dehydrogenase activity at 0.32 M NaCl. Perfect coelution was observed during FPLC on Mono Q (fig.2) and on Superose, and during affinity chromatography on the AH Sepharose 4B column (data not shown). During these steps the molybdenum content increased from 0.046 nmol per mg protein to 3–4 nmol per mg (table 1), whilst the activity per mol

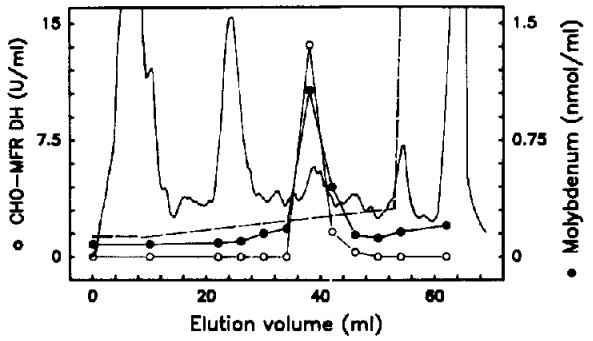

Fig.2. Copurification of molybdenum (•) and formylmethanofuran dehydrogenase activity (○) from *Methanosarcina barkeri* during FPLC on Mono Q HR 10/10. (——) Relative A_{280}; (– – –) NaCl gradient (12 ml of 0.2 M, 40 ml of 0.2–0.4 M, and 16 ml of 2 M). To the Mono Q column the peak activity fractions from the DEAE-Sephacel column were applied.

molybdenum remained essentially constant. The latter finding strongly indicates that formylmethanofuran dehydrogenase is a molybdoenzyme.

An UV/VIS spectrum of the purified formylmethanofuran dehydrogenase is shown in fig.3. It indicates that besides molybdenum the enzyme contains iron-sulfur clusters. The exact content of non-heme iron and acid labile sulfur has not yet been determined. SDS-PAGE revealed the presence of 4 major and 2 minor protein bands. An

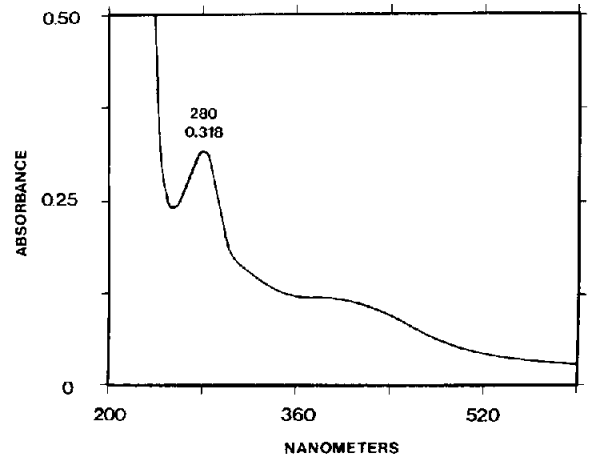

Fig.3. UV/VIS spectrum of purified formylmethanofuran dehydrogenase from *Methanosarcina barkeri*. The enzyme solution (1 ml of 50 mM potassium phosphate, pH 7) contained 0.31 mg protein.

identical subunit pattern was observed for the 200 kDa and the 400 kDa species indicating that the latter is a dimer of the former.

3.2. *Purification of formylmethanofuran dehydrogenase from* Methanobacterium thermoautotrophicum

We tried to purify the enzyme from *Mb. thermoautotrophicum* using an analogous procedure as described for the dehydrogenase from *M. barkeri* (table 1). Several important differences in chromatographic behaviour were observed. Chromatography on DEAE-Sepharose revealed that almost all of the molybdenum present in cell extracts was associated with formylmethanofuran dehydrogenase activity. Upon FPLC on Mono Q three fractions rather than one were obtained containing formylmethanofuran dehydrogenase activity (fig.4). Molybdenum showed an identical elution profile suggesting that also formylmethanofuran dehydrogenase from *Mb. thermoautotrophicum* is a molybdoprotein.

3.3. *The physiological electron acceptor?*

Coenzyme F420 is reduced by formylmethanofuran in cell extracts of *M. barkeri* and of *Mb. thermoautotrophicum* [1]. The purified formylmethanofuran dehydrogenase from *M. barkeri* no longer showed this activity indicating that coenzyme F420 is not the direct physiological electron acceptor.

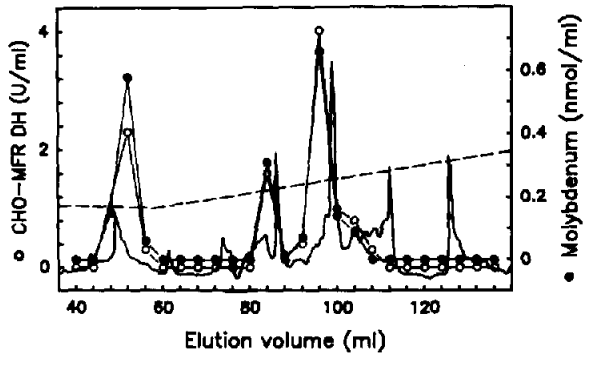

Fig.4. Copurification of molybdenum (●) and formylmethanofuran dehydrogenase activity (○) from *Methanobacterium thermoautotrophicum* during FPLC on Mono Q HR 10/10. (——) Relative A_{280}; (– – –) NaCl gradient (20 ml of 0 M, 20 ml of 0–0.36 M, 20 ml of 0.36 M, 80 ml of 0.36–0.6 M, and 20 ml of 2 M).

4. DISCUSSION

Formylmethanofuran dehydrogenase activity from two representative methanogenic bacteria was found to co-purify with molybdenum. Purified preparations of the enzyme from *Methanosarcina barkeri* contained 3–4 nmol molybdenum per mg protein. The apparent molecular mass of the monomeric form was found to be 200 kDa. From these data a molybdenum content of 0.6–0.8 mol per mol enzyme is calculated suggesting the presence of 1 molybdenum per active site. This stoichiometry is also found for other molybdoenzymes [6].

The molybdenum content of the enzyme was determined by atomic absorption spectroscopy without prior chemical destruction of the enzyme to release the transition metal. It is known that molybdenum can be underestimated under these conditions [16]. Protein was determined by the Bradford method [14] using ovalbumin as standard. This method, as others, yields relative rather than absolute protein values [17]. The molecular mass of the enzyme was estimated from the migration distance after electrophoresis in polyacrylamide gradient gels in relation to molecular mass standards. For some proteins this method gives wrong molecular masses [18]. Last but not least, during purification molybdoproteins tend to lose molybdenum [6,19]. There are thus still some experimental uncertainties with respect to the exact molybdenum content of formylmethanofuran dehydrogenase.

Recently it was shown that in methanogenic bacteria the oxidation of formylmethanofuran is coupled with primary electrogenic sodium translocation [20]. This finding indicates that formylmethanofuran dehydrogenase is involved in energy conservation. Therefore its structure is expected to be complex. Other molybdoenzymes with a direct function in energy conservation are e.g. the dissimilatory nitrate reductase [6,21], dimethyl sulfoxide reductase [19], and trimethylamine *N*-oxide reductase [22] from facultative bacteria and carbon monoxide dehydrogenase from carboxydobacteria [23].

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