Volume 290, number 1,2, 31–34 FEBS 10158 September 1991

ADONIS 001457939100869J

Molybdopterin adenine dinucleotide and molybdopterin hypoxanthine dinucleotide in formylmethanofuran dehydrogenase from *Methanobacterium thermoautotrophicum* (Marburg)

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Received 10 July 1991

Formylmethanofuran dehydrogenase from *Methanobacterium thermoautotrophicum* was purified to apparent homogeneity and found to contain per mol (apparent molecular mass 110 kDa) 0.6 mol molybdenum, 4 mol non-heme iron, 4 mol acid-labile sulfur, and in addition, 0.7 mol of a pterin-containing co-factor (apparent molecular mass 800 Da) which has been characterized. The pterin material was extracted after alkylation by iodoacetamide and the extract subjected to HPLC on Lichrospher 100 RP-18. Three pterin compounds were resolved. On the basis of their UV/visible spectra and of the products formed after cleavage by nucleotide pyrophosphatase and alkaline phosphatase they were identified as the [di(carboxamidomethyl)]-derivatives of molybdopterin guanine dinucleotide (MGD), of molybdopterin adenine dinucleotide (MAD), and of molybdopterin hypoxanthine dinucleotide (MHD). The three pterin dinucleotides were present in the proportions 1:0.4:0.1.

Molybdopterin co-factor; Molybdoenzyme; Formylmethanofuran dehydrogenase; Archaebacterium; Methanogenic bacterium

1. INTRODUCTION

All molybdoenzymes investigated so far, with exception of molybdo-dinitrogenases, contain a pterin co-factor [1], the proposed structure of which is shown in Fig. 1. The co-factor occurs in a mononucleotide and a dinucleotide form, designated as molybdopterin (MPT) and molybdopterin dinucleotide, respectively [2,3]. Molybdopterin, which has a molecular mass of 393 Da, is found in eucaryotes. Molybdopterin dinucleotides, which are larger, have, until now, been found only in procaryotes, both eubacteria and archaebacteria.

Two molybdopterin dinucleotides have been described to date: molybdopterin guanine dinucleotide (MGD) and molybdopterin cytosine dinucleotide (MCD). MGD has been isolated from dimethylsulfoxide reductase of *Rhodobacter sphaeroides* [3], from formylmethanofuran dehydrogenase of *Methanosarcina barkeri* [4] and from formate dehydrogenase of *Methanobacterium formicicum* [5]. MCD has, until now, been found only in carbon monoxide dehydrogenase of *Pseudomonas carboxydoflava* [6].

We report here on the occurrence of two novel dinucleotides in formylmethanofuran dehydrogenase from *Methanobacterium thermoautotrophicum* (Marburg): molybdopterin adenine dinucleotide (MAD) and molybdopterin hypoxanthine dinucleotide (MHD). Formylmethanofuran dehydrogenase catalyzes the first step in the reduction of CO_2 to methane by the anaerobic archaebacterium [7,8].

Abbreviations: MPT, molybdopterin; camMPT, [di(carboxamidomethyl)]-molybdopterin; MGD, molybdopterin guanine dinucleotide; MCD, molybdopterin cytosine dinucleotide; MAD, molybdopterin adenine dinucleotide; MHD, molybdopterin hypoxanthine dinucleotide; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; TBA, tetra-*N*-butylammonium bromide.

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2. MATERIALS AND METHODS

Nucleotide pyrophosphatase (EC 3.6.1.9) Type III (4–8 U/mg) was from Sigma (Deisenhofen, Germany). Alkaline phosphatase (EC 3.1.3.1) (1 U/μl) was from Boehringer Mannheim (Mannheim, Germany). FPLC columns were from Pharmacia (Freiburg, Germany). *M. thermoautotrophicum* (Marburg) (DSM 2133) was from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). The archaebacterium was grown on H_2/CO_2 (80%:20%) as described [9]. Formylmethanofuran dehydrogenase activity, protein, and the contents of molybdenum, non-heme iron, acid-labile sulfur, and pterin were determined as reported previously [10,11]. Formylmethanofuran was synthesized from methanofuran and *p*-nitrophenylformate [12].

Formylmethanofuran dehydrogenase was purified from 25 g freshly harvested cells (wet mass), which were suspended anaerobically in 30 ml 50 mM Tricine/KOH pH 8.0, containing 2 mM DTT. Cell extract was prepared by passing the suspension 3 times through a French pressure cell at 1100×10^5 Pa, followed by centrifugation at $27\,000 \times g$ for 30 min. All subsequent steps in the purification were performed in an anaerobic chamber filled with a gas mixture of 95% N_2 and 5% H_2 and containing palladium catalyst for the removal of O_2 by reduc-

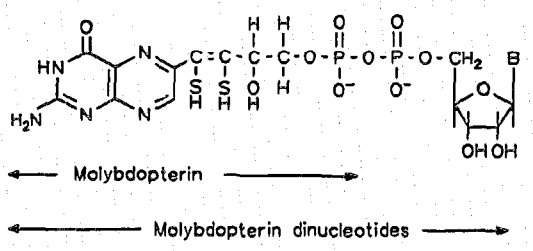

Fig. 1. Proposed structure of molybdopterin and of molybdopterin dinucleotides, both in the oxidized form [2,3]. B = purine or pyrimidine base.

tion with H_2. The 27 000 × *g* supernatant, designated hereafter as cell extract, was applied to a DEAE Sephadex Fast Flow column (2.6 × 12 cm), equilibrated with 50 mM Tricine/KOH pH 8.0, containing 2 mM DTT (2 ml/min). The column was then washed with 200 ml each of the Tricine/DTT buffer containing 0.26 M KCl, 0.31 M KCl, and 0.40 M KCl. Formylmethanofuran dehydrogenase activity eluted in 2 peaks, the first with 0.31 M KCl and the second with 0.40 M KCl. For further purification only the first peak, which contained approximately 60% of the total activity and less contaminating proteins than the second peak, was used. The fractions with enzyme activity were pooled, diluted 1:2 with 50 mM Tricine/KOH pH 8.0, containing 2 mM DTT, and applied to a Mono Q HR 10/10 column equilibrated with the same buffer (4 ml/min). The column was then washed with 40 ml 0.38 M KCl, followed by 80 ml of a 0.38–0.58 M KCl linear gradient in buffer. Enzyme activity was recovered in the 0.40 to 0.46 M KCl fractions. These fractions were pooled and re-applied to a Mono Q HR 10/10 column equilibrated with 50 mM Tris-HCl pH 7.6, containing 2 mM DTT (4 ml/min). The column was washed with 40 ml 0.34 M KCl, followed by 80 ml of a 0.34–0.54 M KCl gradient in buffer. Enzyme activity was recovered in the 0.36 to 0.40 M KCl fractions. These fractions were pooled, supplemented with ammonium sulfate to a final concentration of 1.0 M, and then applied to a Phenyl Superose column HR 10/10 equilibrated with 50 mM Tricine/KOH pH 8.0, containing 2 mM DTT and 1 M ammonium sulfate (1 ml/min). The column was washed with 100 ml of a 1.0 M to 0 M ammonium sulfate linear gradient in buffer. Enzyme activity eluted between 0.70 and 0.64 M ammonium sulfate. The active fractions were pooled, concentrated, and desalted with the help of Centricon-30 microconcentrators (Amicon, Witten, Germany), using 10 mM sodium phosphate pH 7.0 as dilution buffer.

3. RESULTS

3.1. *Purification and properties of formylmethanofuran dehydrogenase*

The enzyme was purified 40-fold to apparent homogeneity in a yield of 27% (Table I). The molecular mass

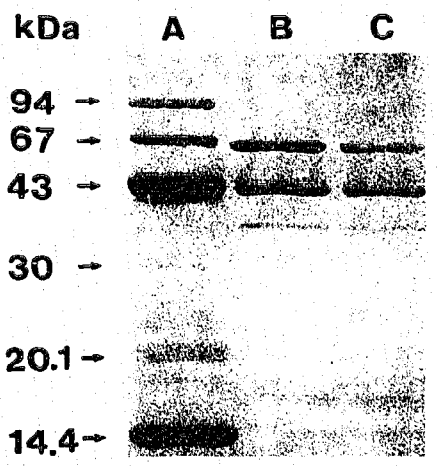

Fig. 2. SDS polyacrylamide gel electrophoresis of formylmethanofuran dehydrogenase from *M. thermoautotrophicum*. Seperation was on 12% polyacrylamide slab gels (8 × 7 cm) which were subsequently stained with silver nitrate. Lanes B and C, 5 μg and 2.5 μg, respectively, of purified enzyme; lane A, molecular mass standards from Pharmacia (Freiburg, Germany) (Electrophoresis calibration kit for low molecular weight proteins).

of the native enzyme was estimated by gel filtration on Superose 12 to be 110 ± 10 kDa. SDS polyacrylamide gel electrophoresis revealed the presence of two polypeptides of apparent molecular mass 60 kDa and 45 kDa, respectively (Fig. 2). When large amounts of protein were applied to the gels, a third polypeptide of apparent molecular mass 38 kDa was observed in most of the preparations. The third polypeptide was less visible when the gels were stained with Coomassie brilliant blue R250 than with silver nitrate. The enzyme contained per mol 0.3–0.6 mol molybdenum, 4 ± 0.5 mol non-heme iron, 4 ± 0.5 mol acid-labile sulfur, and 0.5–0.7 mol of a pterin co-factor of apparent molecular mass 800 Da. The enzyme did not contain FAD or FMN. Double reciprocal plots of the rates vs the substrate concentration yielded an apparent V_{max} of 70 U/mg at pH 8.0 and 65°C, an apparent K_m for formylmethanofuran of 30 μM (at 3 mM methylviologen) and an apparent K_m for methylviologen of 100 μM (at 60

Table I

Purification of formylmethanofuran dehydrogenase from *M. thermoautotrophicum*. Enzyme activity was measured at pH 8.0 by following the reduction of methylviologen (3 mM) with formylmethanofuran (60 μM) at 578 nm. One U = 2 μmol methylviologen reduced/min.

Purification step	Activity (U)	Protein (mg)	Specific activity (U/mg)	Molybdenum (nmol)	Activity per molybdenum (U/nmol)
Cell extract	3347	2100	1.6	385.0	8.7
DEAE Sephadex Fast Flow	2161	538	4.0	171.8	12.6
Mono Q HR 10/10 (pH 8.0)	1241	65	19.1	82.8	15.0
Mono Q HR 10/10 (pH 7.6)	955	26	36.7	67.6	14.1
Phenyl Superose HR 10/10	921	14.4	64.0	52.0	17.7

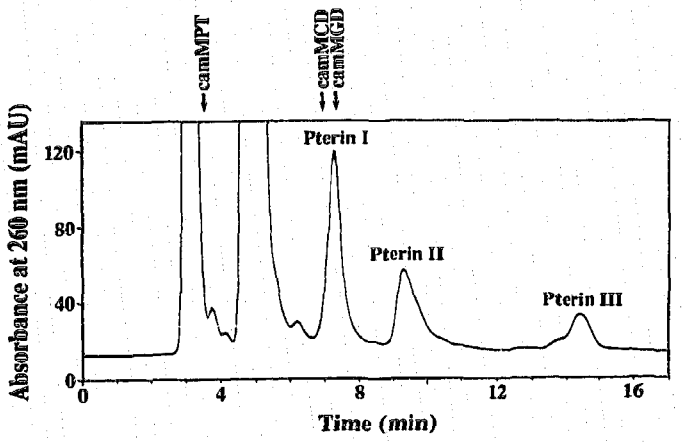

Fig. 3. HPLC elution profile of the compounds extracted from purified formylmethanofuran dehydrogenase by SDS in the presence of iodoacetamide. The compounds were separated on a Lichrospher 100 RP-18 column (0.4 × 12.5 cm) using 50 mM ammonium acetate pH 6.8, containing 3% methanol as eluant. The flow rate was 1 ml/min. The absorbance at 260 nm was followed using the detection system described in Fig. 4. The arrows indicate where camMPT, camMCD, and camMGD would have eluted.

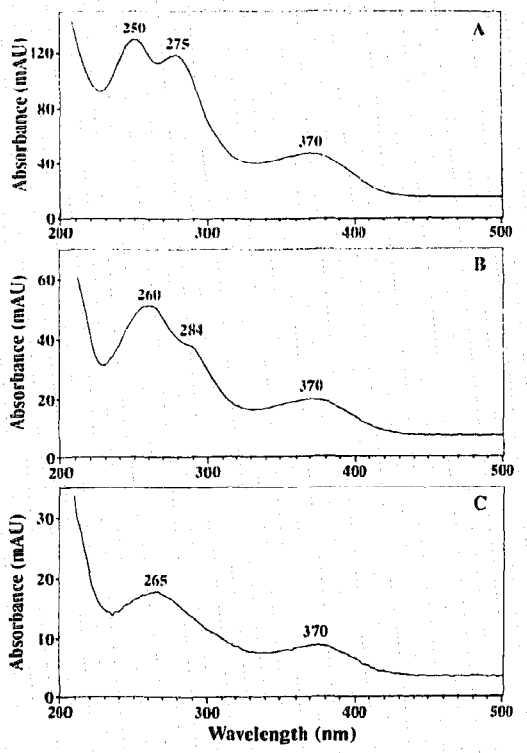

Fig. 4. UV/visible spectra (A) of pterin I, (B) of pterin II, and (C) of pterin III after separation by HPLC as shown in Fig. 3. The spectra were recorded on-line via a Hewlett Packard 1040 A/1090 A diode array detector connected to a Hewlett Packard compactcomputer HP 85-B.

μM formylmethanofuran). The pH optimum was at pH 8.0 and the temperature optimum at 70°C. The Arrhenius activation energy at 50°C was 47.6 kJ/mol.

3.2. *Evidence for the presence of three different pterins in the purified enzyme*

To identify the pterin co-factor purified formylmethanofuran dehydrogenase was extracted under reducing conditions in the presence of iodoacetamide and SDS as described previously [4]. This procedure yields the [di(carboxamidomethyl)]-derivatives, abbreviated by the prefix cam. The extract was subjected to HPLC on Lichrospher 100 RP-18 (Merck, Darmstadt, Germany). The elution profile is shown in Fig. 3. Three of the five separated compounds displayed UV/visible spectra indicative for a pterin-containing compound (Fig. 4). They are designated pterin I, II, and III. The two compounds which eluted first had uncharacteristic UV/visible spectra with an absorbance maximum at 225 nm and 260 nm, respectively (data not shown) and are not considered to be pterins.

Pterin I had an UV/visible spectrum (Fig. 4A) and a retention time (Fig. 3) identical to those of [di(carboxamidomethyl)]-MGD (camMGD). Hydrolysis in the presence of nucleotide pyrophosphatase and alkaline phosphatase yielded dephospho-camMGD [3] and guanosine in stoichiometric amounts (results not shown), indicating that pterin I is indeed camMGD. Pterin II and III exhibited UV/visible spectra (Fig. 4B and C) and retention times (Fig. 3) different from those of camMPT, camMGD, and camMCD. As judged from the absorbance difference at 370 nm the three pterins were present in the proportions 1 to 0.4 to 0.1.

3.3. *Identification of pterin II as camMAD*

Pterin II was incubated at pH 6.8 in the presence of nucleotide pyrophosphatase and the hydrolysis products were separated by HPLC on Lichrospher 100 PR-18 equilibrated and eluted at a rate of 1 ml/min with 22 ml 50 mM ammonium acetate pH 6.8, containing 0.5 mM tetra *N*-butylammonium bromide (TBA), and then with 50 mM ammonium acetate pH 6.8, containing 0.5 mM TBA and 50% methanol. The product which eluted first was identified as AMP by its retention time (20.2 min) and its UV/visible spectrum. The product which eluted second exhibited a spectrum and a retention time (24.3 min) identical to those of camMPT. In a further experiment pterin II was incubated at pH 6.8 in the presence of both nucleotide pyrophosphatase and alkaline phosphatase. The hydrolysis products were separated by HPLC on Lichrospher 100 RP-18 equilibrated with 50 mM ammonium acetate pH 6.8 containing 10% methanol and eluted with the same buffer at a rate of 1 ml/min. The two products showed retention times and UV/visible spectra identical to those of adenosine (9.3 min) and dephospho-camMPT (3.3 min). The data strongly indicate that pterin II is camMAD.

3.4. *Identification of pterin III as camMHD*

Pterin III was incubated at pH 6.8 in the presence of

both nucleotide pyrophosphatase and alkaline phosphatase, and the hydrolysis products were separated by HPLC on Lichrospher 100 RP-18 equilibrated and eluted at a rate of 1 ml/min with 50 mM ammonium acetate pH 6.8, containing 0.5 mM tetra *N*-butylammonium bromide. The compound which eluted first was identified as IMP by its retention time (9.1 min) and its UV/visible spectrum with a characteristic maximum at 249 nm. The product which eluted second exhibited a spectrum and a retention time (10.3 min) identical to those of dephospho-camMPT. The results indicate that pterin III is camMHD.

4. DISCUSSION

The finding that purified formylmethanofuran dehydrogenase from *M. thermoautotrophicum* contains 3 different molybdopterin dinucleotides was rather unexpected. Two explanations can be envisaged: (i) the purified enzyme consists of a mixture of 3 isoenzymes to each of which a different dinucleotide is bound, or (ii) the purified protein part of the enzyme is homogeneous, but heterogeneous with respect to the co-factor bound. Until now, all attempts to resolve the purified enzyme into isoenzymes failed, suggesting, but not proving, that the enzyme preparation analyzed was homogeneous.

Formylmethanofuran dehydrogenase from *Methanobacterium wolfei*, a close relative of *M. thermoautotrophicum*, only contains MGD (R.A. Schmitz, unpublished results) and so does the enzyme from *M. barkeri* [4]. More than 60% of the pterin co-factor in formylmethanofuran dehydrogenase from *M. thermoautotrophicum* was found to be MGD and only 25% and 10%, respectively, to be MAD and MHD. As is the case for MGD, the latter 2 compounds are also dinucleotides of molybdopterin with a purine nucleotide rather than with a pyrimidine nucleotide. The most likely interpretation of the results is therefore that in *M. thermoautotrophicum* the enzyme catalyzing the synthesis of MGD from MPT and GTP is not completely specific for GTP and can also use other purine nucleoside triphosphates, namely ATP and ITP, as substrates.

Formylmethanofuran dehydrogenase from *M. thermoautotrophicum* was found to be composed of two subunits of apparent molecular masses 60 kDa and 45 kDa, respectively, and to contain 4 mol non-heme iron and 4 mol acid-labile sulfur per mol enzyme of apparent molecular mass 110 kDa. The enzyme from *M. barkeri* has been shown to be composed of 6 subunits of apparent molecular masses 65 kDa, 50 kDa, 37 kDa, 34 kDa, 29 kDa and 17 kDa, and to contain 28 mol non-heme iron and 28 mol acid-labile sulfur per mol enzyme of apparent molecular mass 220 kDa [11]. It therefore has to be considered that formylmethanofuran dehydrogenase from *M. thermoautotrophicum* is also a more complex enzyme which loses some of its subunits during purification.

Acknowledgements: This work was supported by a grant from the Deutsche Forschungsgemeinschaft and from the Fonds der Chemischen Industrie.

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