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The molybdenum cofactor of formylmethanofuran dehydrogenase from *Methanosarcina barkeri* is a molybdopterin guanine dinucleotide

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The molybdenum cofactor of formylmethanofuran dehydrogenase from methanol-grown *Methanosarcina barkeri* was isolated as the [di(carboxamidomethyl)]-derivative. The alkylated factor showed an absorption spectrum and chemical properties identical to those recently reported for the molybdenum cofactor of dimethyl sulfoxide reductase from *Rhodobacter sphaeroides*. By treatment with nucleotide pyrophosphatase the factor was resolved into two components, which were identified as [di(carboxamidomethyl)]-molybdopterin and GMP by their absorption spectra, their retention times on Lichrospher RP-18, and by their conversion to dephospho-[di(carboxamidomethyl)]-molybdopterin and guanosine, respectively, in the presence of alkaline phosphatase. The GMP-moiety was sensitive to periodate, identifying it as the 5'-isomer. These results demonstrate that the molybdenum cofactor isolated from formylmethanofuran dehydrogenase contains the phosphoric anhydride of molybdopterin and 5'-GMP.

Molybdopterin cofactor; Formylmethanofuran dehydrogenase; Methanogenic archaebacteria; *Methanosarcina barkeri*

1. INTRODUCTION

Formylmethanofuran dehydrogenase is a molybdoenzyme [1,2] involved in the energy metabolism of methanogenic archaebacteria [3,4]. Besides molybdenum the enzyme contains a pterin cofactor of apparent molecular mass 780 Da [5]. A pterin cofactor of this size has first been reported to be present in carbon monoxide dehydrogenase from *Pseudomonas carboxydoflava* [6,7] and was later found in many other eubacterial molybdoenzymes [8,9]. Whereas the pterin cofactor of eucaryotic molybdoenzymes has been reported to have an apparent molecular mass of only 340 Da [10].

The 340 Da pterin found in eucaryotes is designated molybdopterin (MPT) (Fig. 1A) [11]. It is isolated in the presence of iodoacetamide as the [di(carboxamidomethyl)]-derivative (camMPT) (Fig. 1B). The structure of camMPT has been elucidated in 1987 by Kramer et al. [12]. It confirmed the structure proposed earlier for MPT [13].

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Abbreviations: MPT, molybdopterin; camMPT, [di(carboxamidomethyl)]-molybdopterin; MGD, molybdopterin guanine dinucleotide; camMGD, [di(carboxamidomethyl)]-molybdopterin guanine dinucleotide; MCD, molybdopterin cytosine dinucleotide; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate

The larger pterin found in eubacteria is composed of molybdopterin, an additional phosphate, and an aromatic residue. This was known since 1986 by the work of Meyer and collaborators [6,7,14]. The structure of the compound remained, however, obscure until recently. Johnson et al. [15] reported that the pterin cofactor of dimethyl sulfoxide reductase from *Rhodobacter sphaeroides* contains molybdopterin and 5'-GMP connected via a pyrosphosphate bond. The novel cofactor was, in analogy to FMN and FAD, designated molybdopterin guanine dinucleotide (MGD). The structures of MGD and of its [di(carboxamidomethyl)]-derivative (camMGD) are shown in Fig. 1C,D.

In the meantime the structure of the pterin cofactor of carbon monoxide dehydrogenase from *P. carboxydoflava* has also been determined [16]. Surprisingly the prosthetic group of this enzyme turned out to be a molybdopterin cytosine dinucleotide (MCD). It thus appears that different molybdoenzymes contain different molybdopterin dinucleotides.

We report here that the molybdenum pterin cofactor of formylmethanofuran dehydrogenase from the archaebacterium *Methanosarcina barkeri* is a molybdopterin guanine dinucleotide and thus identical to that of dimethyl sulfoxide reductase from the eubacterium *R. sphaeroides*.

2. MATERIALS AND METHODS

Iodoacetamide was from Merck-Schuchard (Darmstadt, FRG).

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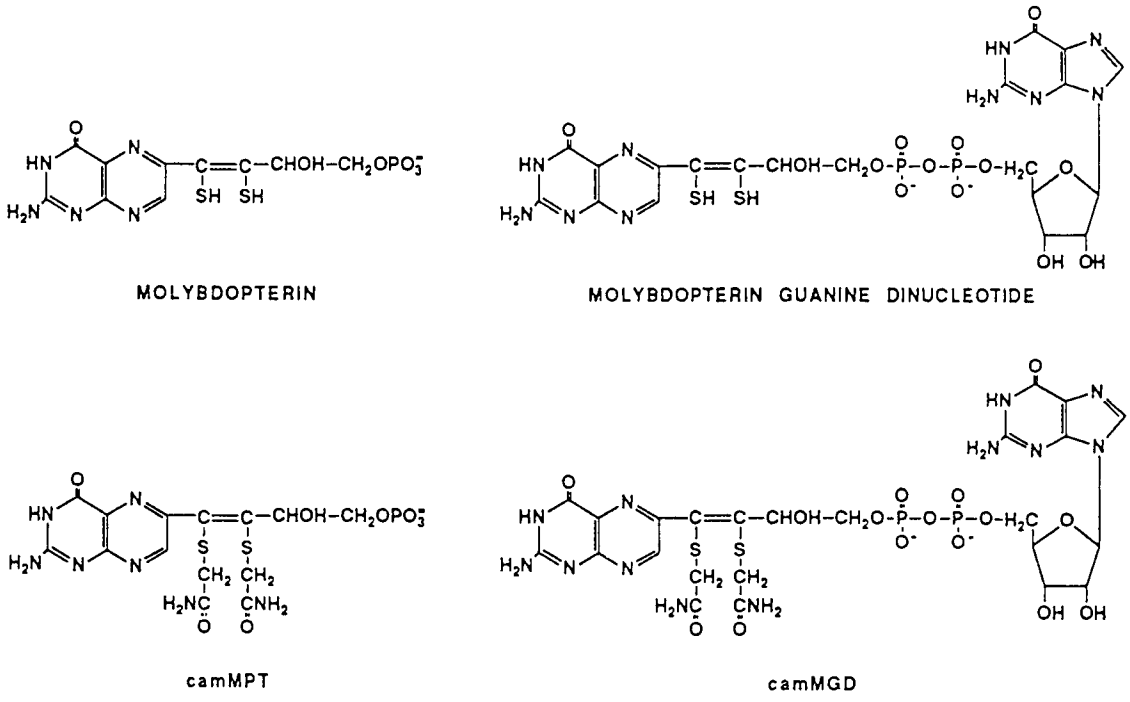

Fig. 1. Proposed structure of the oxidized forms of (A) molybdopterin (MPT); (B) [di(carboxamidomethyl)]-molybdopterin (camMPT); (C) molybdopterin guanine dinucleotide (MGD); and (D) [di(carboxamidomethyl)]-molybdopterin guanine dinucleotide (camMGD) [15].

Xanthine oxidase (EC 1.1.3.22) (1 U/mg) and periodic acid were from Serva (Heidelberg, FRG), nucleotide pyrophosphatase (EC 3.6.1.9) Type III (4–8 U/mg) and guanosine 5'-monophosphate were from Sigma (Deisenhofen, FRG). Alkaline phosphatase (EC 3.1.3.1) (1 U/μl) was from Boehringer-Mannheim (Mannheim, FRG). Formylmethanofuran dehydrogenase from *M. barkeri* (strain Fusaro) (DSM 804) was purified as described [2].

The molybdenum cofactor of formylmethanofuran dehydrogenase from *M. barkeri* was isolated as the [di(carboxamidomethyl)]-derivative. The initial purification steps were performed in an anaerobic chamber (Coy, Ann Arbor, USA). Four mg iodoacetamide were added to 4 mg purified enzyme in 2 ml of an anaerobic 10 mM sodium phosphate buffer, pH 7.0. Then 25 μl of a 100 mM sodium dithionite solution was added followed by 25 mg SDS. The 8 ml vial was closed with a rubber stopper and incubated for two hours in a water bath at 37°C. After overnight incubation at room temperature the reaction mixture was subjected to ultrafiltration using Centricon-10 microconcentrators (Amicon, Witten, FRG). The filtrate was concentrated by lyophilization, dissolved in 10 mM acetic acid, and desalted on a Sephadex G15 column (44 × 1 cm) equilibrated with 10 mM acetic acid. Fractions containing pterin were identified by their characteristic fluorescence (380 nm excitation, 460 nm emission) after alkalization of fraction aliquots with NaOH. The pterin-containing fractions were neutralized with NH_3 and concentrated by rotoevaporation. Final purification of the [di(carboxamidomethyl)]-derivative was achieved by chromatography on a C-18 HPLC column (125 × 4 mm, Lichrospher 100 RP-18 from Merck, Darmstadt, FRG) using 50 mM ammonium acetate, pH 6.8, containing 3% (v/v) methanol as eluent. Detection was via a Hewlett-Packard 1040A/1090A diode array detector connected to a Hewlett-Packard compactcomputer HP 85-B. Elution profiles were recorded at 280 nm. All absorption spectra were obtained on-line.

Enzymatic hydrolysis of the purified [di(carboxamidomethyl)]-derivative was achieved by incubation in 50 mM ammonium acetate, pH 6.8, containing 10 mM $MgCl_2$ in the presence of nucleotide pyrophosphatase (10 U) and, where indicated, also of alkaline phosphatase (10 U). The 150 μl reaction mixtures were incubated at room temperature for 30 min and then aliquots (30–70 μl) analyzed for product formation by HPLC as described above.

The molybdenum cofactor of milk xanthine oxidase was isolated as the [di(carboxamidomethyl)]-derivative essentially as described for the cofactor of formylmethanofuran dehydrogenase. The compound obtained showed a UV/VIS spectrum identical to that published for camMPT [15].

3. RESULTS

When purified formylmethanofuran dehydrogenase is extracted with guanidine hydrochloride two fluorescent compounds are obtained, one with an apparent molecular mass of 780 Da and the other with 430 Da. Both compounds are converted to pterin 6-carboxylic acid by oxidation with permanganate [5]. We now found that the 780 Da pterin is converted to the 430 Da pterin by treatment with nucleotide pyrophosphatase, an enzyme known to catalyze the cleavage of the pyrophosphate linkage of, example given, FAD or NAD. These findings indicated that the molybdenum cofactor of formylmethanofuran dehydrogenase is most probably a dinucleotide of molybdopterin and a nucleotide monophosphate as is the molybdenum cofactor of dimethyl sulfoxide reductase from *R. sphaeroides* [15] and of carbon monoxide dehydrogenase from *P. carboxydoflava* [16].

3.1. *Purification of the [di(carboxamidomethyl)]-derivative of the molybdenum cofactor*

The molybdenum cofactor of formylmethanofuran dehydrogenase from *M. barkeri* was isolated as the

[di(carboxamidomethyl)]-derivative essentially as described by Johnson et al. [15]. To an anaerobic sample of the purified enzyme (4 mg protein) iodoacetamide and then sodium dodecyl sulfate were added at pH 7. After anaerobic incubation for 2 h at 37°C and then for 12 h at room temperature protein was removed by ultrafiltration and the filtrate was lyophilized. The lyophilisate was dissolved in 10 mM acetic acid and applied to a Sephadex G15 column for the separation of the [di(carboxamidomethyl)]-derivative from low molecular weight compounds (iodoacetamide, SDS, buffer). The fractions containing the [di(carboxamidomethyl)]-derivative were pooled, neutralized with NH_3, and concentrated by rotoevaporation. The slightly yellow residue was dissolved in 0.5 ml 50 mM ammonium acetate, pH 6.8, containing 3% (v/v) methanol. Aliquots (0.05–0.08 ml) of the solution were subjected to HPLC on Lichrospher 100 RP-18. The elution profile is given in Fig. 2. The UV/VIS spectra of the 4 separated compounds are given in the inset.

The compounds in peaks 1–3 did not show a pterin spectrum and are therefore not considered to be components of the molybdenum cofactor. The compound which eluted in peak 4, however, exhibited a UV/VIS spectrum characteristic for a [di(carboxamidomethyl)]-molybdopterin dinucleotide. This compound was therefore re-chromatographed on Lichrospher 100 RP-18. It eluted in only 1 peak showing a UV/VIS spectrum resembling very closely that published for camMGD (Fig. 3).

3.2. *Identification of camMPT and 5'-GMP as products formed in the presence of nucleotide pyrophosphatase*

From the spectrum shown in Fig. 3 the nucleotide moiety of the molybdenum cofactor cannot definitively be identified. We therefore incubated the [di(carboxamidomethyl)]-derivative at pH 6.8 in the presence of

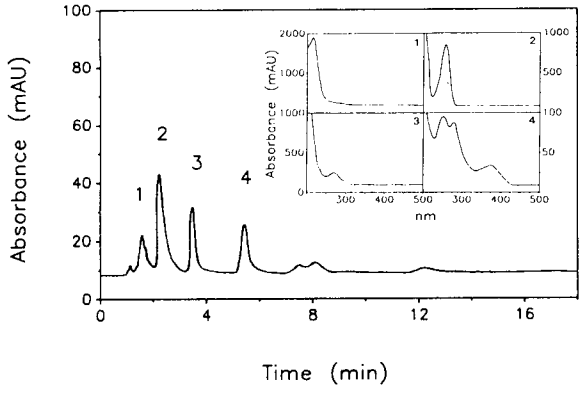

Fig. 2. HPLC elution profile of the compounds extracted from formylmethanofuran dehydrogenase in the presence of iodoacetamide. The insets show the UV/VIS spectra of the 4 compounds resolved.

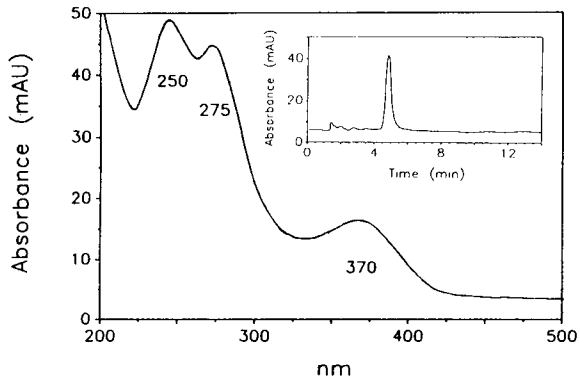

Fig. 3. UV/VIS spectrum of the [di(carboxamidomethyl)]-derivative (peak 4 in Fig. 2) after re-chromatography on Lichrospher 100 RP-18. The inset shows the elution profile.

nucleotide pyrophosphatase and separated the hydrolysis products by HPLC on Lichrospher 100 RP-18 (Fig. 4). The product, which eluted first, showed the spectrum given in Fig. 4A. It was identified as GMP by its retention time and its characteristic UV/VIS spectrum, which were identical to those of authentic GMP. The product, which eluted second, exhibited the spectrum given in Fig. 4B. It was identified as camMPT by

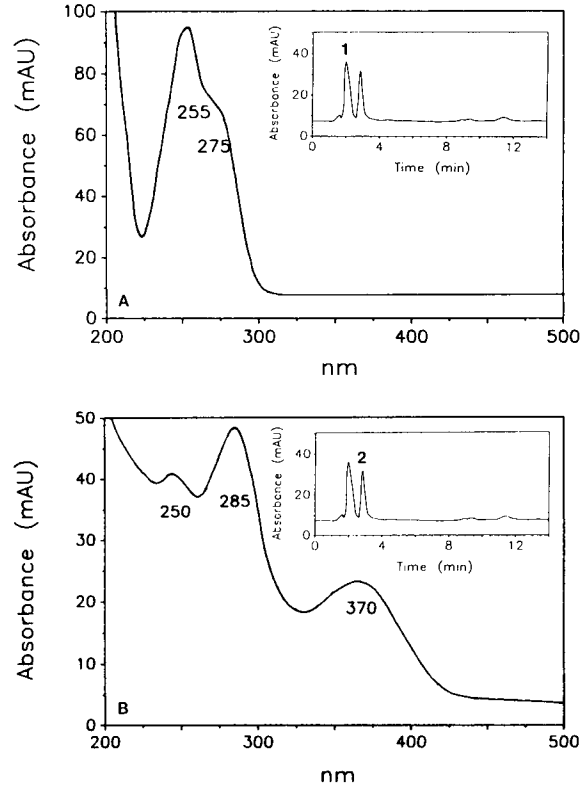

Fig. 4. UV/VIS spectra of the two degradation products formed from the [di(carboxamidomethyl)]-derivative in the presence of nucleotide pyrophosphatase. The products were separated by HPLC on Lichrospher 100 RP-18. The insets show the elution profile. (A) Product 1 was identified as GMP; (B) product 2 was identified as camMPT (see text).

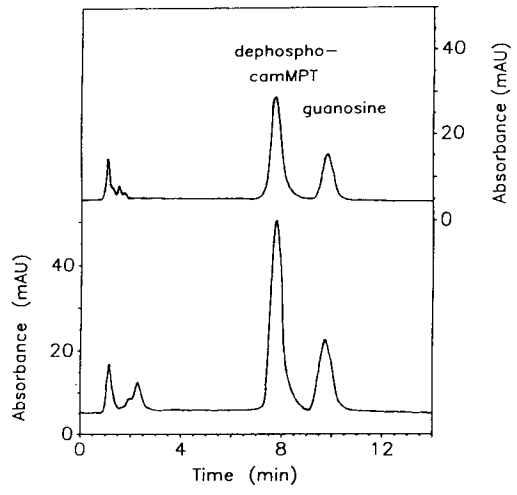

Fig. 5. HPLC elution profile (lower trace) of the products formed from the [di(carboxamidomethyl)]-derivative in the presence of nucleotide pyrophosphatase and alkaline phosphatase. The upper trace shows the elution profile of authentic dephospho-camMPT and of guanosine.

its retention time and UV/VIS spectrum. The camMPT, which was used as reference substance, was isolated from milk xanthine oxidase after alkylation with iodoacetamide [12,15]. The data strongly indicate that the molybdenum cofactor of formylmethanofuran dehydrogenase from *M. barkeri* is a molybdopterin guanine dinucleotide.

In a further experiment we incubated the [di(carboxamidomethyl)]-dinucleotide at pH 6.8 in the presence of both nucleotide pyrophosphatase and alkaline phosphatase and separated the hydrolysis products by HPLC on Lichrospher 100 RP-18 (Fig. 5). The two products showed retention times and UV/VIS spectra (data not shown) characteristic for guanosine and dephospho-camMPT. This finding further substantiates the fact that the molybdenum cofactor of formylmethanofuran dehydrogenase is composed of molybdopterin and guanosine monophosphate connected via a pyrophosphate bond.

The GMP moiety was identified as 5'-GMP by incubation in the presence of periodate (0.1 mM) at pH 4.5. The nucleotide was degraded as evidenced by HPLC (data not shown). Only 5'-nucleotides react with periodate under these conditions.

4. DISCUSSION

The available data indicate that at least 3 different molybdenum pterin cofactors exist: (i) molybdopterin (Fig. 1A) which is the prosthetic group of the eucaryotic molybdoenzyme xanthine oxidase, aldehyde oxidase, sulfite oxidase, and nitrate reductase [17,18]; (ii) molybdopterin guanine dinucleotide found in dimethyl sulfoxide reductase from *R. sphaeroides* [15] and in formylmetanofuran dehydrogenase from *M. barkeri*; and (iii) molybdopterin cytosine dinucleotide found in carbon monoxide dehydrogenase from *P. carboxydoflava* [16]. Many other molybdoenzymes with a pterin cofactor are known. They have, however, not yet been analyzed: formate dehydrogenase, nitrate reductase [19], dimethyl sulfoxide reductase [20], trimethylamine N-oxide reductase [21], biotin sulfoxide reductase [17], nicotine dehydrogenase [22], nicotinic acid dehydrogenase, 6-hydroxynicotinic acid dehydrogenase [8,23], 2-furoyl-CoA dehydrogenase [24], arsenate reductase [17], picolinate dehydrogenase [25], and xanthine dehydrogenase [26] from eubacteria, and formate dehydrogenase from the archaebacterium *Methanobacterium formicicum* [27]. Some of these enzymes may turn out to contain a molybdopterin dinucleotide with a nucleotide other than GMP or CMP. This remains, however, to be demonstrated.

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