Formylmethanofuran: tetrahydromethanopterin formyltransferase from *Methanosarcina barkeri*

Identification of N^5-formyltetrahydromethanopterin as the product

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Formylmethanofuran: tetrahydromethanopterin formyltransferase was purified from methanol grown *Methanosarcina barkeri* to apparent homogeneity and characterized with respect to its molecular and kinetic properties. The enzyme was found to be very similar to the formyltransferase from H_2/CO_2 grown *Methanobacterium thermoautotrophicum*. It also catalyzed the formation of N^5-formyltetrahydromethanopterin rather than of N^{10}-formyltetrahydromethanopterin from formylmethanofuran and tetrahydromethanopterin.

Methanofuran; Formylmethanofuran; Tetrahydromethanopterin; Formyltetrahydromethanopterin; *Methanosarcina barkeri*; *Methanobacterium thermoautotrophicum*

1. INTRODUCTION

N-Formyltetrahydromethanopterin (formyl-H_4MPT) is an intermediate in the energy metabolism of methanogenic archaebacteria [1]. During growth on CO_2 and H_2 this compound is formed from formylmethanofuran (formyl-MFR) and tetrahydromethanopterin (H_4MPT) in reaction 1 and it is converted to N^5,N^{10}-methenyl-H_4MPT in reaction 2. During growth on methanol the reverse reactions are operative [2,3]. It has been proposed that in some methanogens N^5-formyl-H_4MPT and in others the N^{10}-isomer is the intermediate [4].

(1) Formyl-MFR + H_4MPT $\rightleftharpoons$ Formyl-H_4MPT + MFR

(2) Formyl-H_4MPT + H^+ $\rightleftharpoons$ Methenyl-H_4MPT$^+$

Reactions 1 and 2, which are slightly exergonic under standard conditions [5], are catalyzed by formylmethanofuran:tetrahydromethanopterin formyltransferase and by N^5,N^{10}-methenyltetrahydromethanopterin cyclohydrolase, respectively. The two enzymes have been purified from *Methanobacterium thermoautotrophicum* (strain ΔH) grown on H_2 and CO_2 and have both been shown to be specific for N^5-formyl-H_4MPT [6,7]. The cyclohydrolase has also been purified from methanol grown *Methanosarcina barkeri* (strain MS) [4]. The analyses of the product formed from N^5,N^{10}-methenyl-H_4MPT by the enzyme from *M. barkeri* were interpreted to indicate that the cyclohydrolase from this organism is specific for the N^{10}-isomer. The evidence was, however, not unambiguous and experiments showing that the enzyme can catalyze the conversion of N^{10}-formyl-H_4MPT to N^5,N^{10}-methenyl-H_4MPT were not presented [4].

If in *M. barkeri* the cyclohydrolase is specific for N^{10}-formyl-H_4MPT then the formyltransferase from this organism should also use the N^{10}-isomer. We therefore purified the formyltransferase from methanol grown *M. barkeri* and determined the *N*-formyl-H_4MPT isomer generated. We also tested whether cell extracts of *M. barkeri* are capable of catalyzing the conversion of N^{10}-formyl-H_4MPT to N^5,N^{10}-methenyl-H_4MPT.

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Abbreviations: MFR, methanofuran; H_4MPT, tetrahydromethanopterin; MES, β-morpholinoethane sulfonic acid; MOPS, γ-morpholinopropane sulfonic acid; Tricine, *N*-[Tris(hydroxymethyl)methyl]glycine; 1 U = 1 μmol/min

2. MATERIALS AND METHODS

M. barkeri (strain Fusaro, DSM 804) and *M. thermoautotrophicum* (strain Marburg, DSM 2133) were from the Deutsche Sammlung von Mikroorganismen (Braunschweig, FRG). The two bacteria were grown on methanol and H_2/CO_2, respectively, and harvested and stored as described [8].

MFR and H_4MPT were isolated from *M. thermoautotrophicum* (strain Marburg) [9]. Formyl-MFR was synthesized from MFR and 4-nitrophenylformate [6]. N^5-formyl-H_4MPT was generated from formylmethanofuran and H_4MPT with purified formyltransferase

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Table I

Purification of formylmethanofuran:tetrahydromethanopterin formyltranferase from *Methanosarcina barkeri*

Purification step	Activity[a]	Protein[b]	Specific activity	Purification	Yield
	(U)	(mg)	(U/mg)	(-fold)	(%)
Cell extract	953	762	1.25	1	100
60% $(NH_4)_2SO_4$-supernatant	612	300	2.04	1.63	64
Phenylsepharose	483	7.2	67.6	54.1	51
Mono Q HR 10/10	385	0.63	610	488	41

[a] The activity was determined at pH 7, 37°C and non-saturating substrate concentrations (0.1 mM formylmethanofuran; 0.065 mM H_4MPT); 1 U = 1 μmol · min^{-1}.
[b]Protein was determined via the Bradford method [15,16] using ovalbumin as standard.

from *M. thermoautotrophicum* [6]. N^{10}-formyl-H_4MPT was chemically synthesized from N^5,N^{10}-methenyl-H_4MPT at pH 14 [4;10]. N^5,N^{10}-methenyl-H_4MPT was prepared from H_4MPT and formaldehyde in the presence of purified N^5,N^{10}-methylene-H_4MPT dehydrogenase from *M. thermoautotrophicum* [11,12].

2.1. *Purification of formyltransferase from* M. barkeri

Cell suspensions (13 ml; 40 mg protein/ml) in 50 mM MOPS/KOH pH 7.0 were passed twice through a French pressure cell at 120 MPa and room temperature under anaerobic conditions. After centrifugation at 27 000 × *g* at 4°C for 30 min the supernatant (designated cell extract) was supplemented under aerobic conditions with saturated ammonium sulfate solution in 50 mM Tricine/KOH pH 8.0 at 0°C to give a final concentration of 60% saturation. After an hour the precipitated protein was removed by 30 min centrifugation at 27 000 × *g*. The supernatant was applied to a column (4 × 5 cm) of Phenylsepharose CL-4B (Pharmacia, Freiburg, FRG) equilibrated with 50 mM Tricine/KOH pH 8. The column was subsequently washed with 200 ml of 50 mM Tricine/KOH pH 8, 200 ml buffer containing 20% ethylene glycol and 200 ml buffer containing 40% ethylene glycol. The formyltransferase activity was recovered in the 40% ethylene glycol fraction. After dialysis against 50 mM Tricine/KOH pH 8 the enzyme was further purified by ion exchange chromatography on Mono Q HR 10/10 (Pharmacia, Freiburg, FRG) previously equilibrated with 50 mM Tricine/KOH pH 8 using a linear gradient of 0–2 M KCl (800 ml). The formyltransferase activity eluted at 0.9 M KCl in 12 ml. After concentration and desalting by ultrafiltration the enzyme in 50 mM Tricine/KOH pH 8 could be stored at 4°C without significant loss in activity for at least 1 week.

2.2. *Purification of formyltransferase from* M. thermoautotrophicum

The formyltransferase from the Marburg strain was purified almost 300-fold in a yield of 64% and a specific activity of 300 U/mg as described by Donnelly and Wolfe [6]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed the presence of only one protein band at the 39.5 kDa position. The properties of the formyltransferase from *M. thermoautotrophicum* (strain Marburg) were found to be almost identical to those described for the enzyme from *M. thermoautotrophicum* (strain ΔH) which reflects that the two strains are phylogenetically closely related [13].

2.3. *Partial purification of cyclohydrolase from* M. thermoautotrophicum

Cyclohydrolase was partially purified by chromatography on a DEAE-Sepharose column (fast flow) (Pharmacia, Freiburg, FRG) which was eluted with a step NaCl gradient (0-2 M) in 50 mM Tricine/KOH pH 8 under anaerobic conditions. The cyclohydrolase activity eluted at a NaCl concentration of 0.4 M NaCl. Using this procedure a 3.5-fold purification was achieved yielding a cyclohydrolase preparation with a specific activity of 5 U/mg which was completely devoid of formyltransferase activity.

2.4. *Assay for formyltransferase activity*

The activity was routinely assayed in the direction of formyl-H_4MPT formation from formyl-MFR. The assays were performed in 1 ml 0.5 cm anaerobic cuvettes at 37°C (*M. barkeri*) or 65°C (*M. thermoautotrophicum*). If not indicated otherwise the 0.7 ml reaction mixture contained: 20 mM potassium phosphate pH 7.0; 2 mM dithiothreitol; 0.1 mM formyl-MFR; 0.065 mM H_4MPT and formyltransferase. The reaction was started by the addition of the enzyme and was followed photometrically by measuring the increase in absorbance at 282 nm ($\Delta\varepsilon = 5.1$ mM^{-1} · cm^{-1}) due to the formation of N^5-formyl-H_4MPT [6].

3. RESULTS AND DISCUSSION

3.1. *Purification and properties of the enzyme*

The formyltransferase from *M. barkeri* was purified almost 500-fold to a specific activity of 610 U/mg in a 40% yield (Table I). The purification procedure was essentially that described for the formyltransferase from *M. thermoautotrophicum* [6]. The two enzymes showed almost identical purification properties. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the formyltransferase from *M. barkeri* revealed the presence of only one polypeptide of apparent molecular mass 32 kDa (Fig. 1). Via gel filtration on Bio Sil TSK 250 (Bio-Rad Laboratories, Richmond, CA) the apparent molecular mass of the native enzyme was determined to be 35 kDa using bovine serum albumin (67 kDa), ovalbumin (45 kDa), chymotrypsin (25 kDa), and cytochrome c (12.3 kDa) as standards. The UV/Vis spectrum of the purified formyltransferase was identical to that of bovine serum albumin indicating the absence of a chromophoric prosthetic group. Almost identical properties have been reported for the formyltransferase from *M. thermoautotrophicum* (strain ΔH) [6,14]. Differences in apparent molecular masses of the subunits, however, exist. The enzyme from *M. thermoautotrophicum* shows a subunit apparent molecular mass of 41 kDa [6] and the enzyme from *M. barberi* of 32 kDa (see above). In-

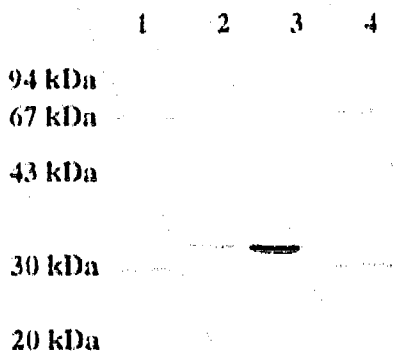

Fig. 1. Analysis of purified formyltransferase from *M. barkeri* by sodium dodecyl sulfate polyacrylamide (12.5%) gel electrophoresis. Lane 2 and 3, 2.1 μg and 5.3 μg purified enzyme; Lane 1 and 4, molecular mass standards, 3 μg each of phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carboanhydrase (30 kDa), and soybean trypsin inhibitor (20 kDa). The gel was stained with Coomassie brilliant blue R250.

terestingly, the molecular mass deduced from the amino acid sequence of the formyltransferase from *M. thermoautotrophicum* is 31.4 kDa [14].

The formyltransferase from *M. barkeri* exhibited a pH optimum at 7.0 in potassium phosphate buffer and a temperature optimum at 65°C. At this temperature the enzyme was completely stable for more than 4 h. The activity was also stable in the presence of molecular oxygen. The formyltransferase could therefore be purified under aerobic conditions, a property also shared by formyltransferase from *M. thermoautotrophicum* (the enzyme activity had, however, to be tested under strictly anaerobic conditions since H_4MPT rapidly auto-oxidizes in the presence of O_2).

Initial velocity studies revealed Michaelis-Menten kinetics. Reciprocal plots of $1/v$ versus $1/[S]$ at different fixed concentrations of the second substrate yielded straight lines which were parallel. From a replot of the intercepts of the ordinate versus the reciprocal substrate concentration a V_{max} (37°C) of 3700 U/mg ($k_{cat} = 1973\ s^{-1}$), a K_m for H_4MPT of 0.4 mM and a K_m for formyl-MFR of 0.4 mM were obtained. With purified formyltransferase from *M. thermoautotrophicum* (strain Marburg) a V_{max} (65°C) of 4200 U/mg ($k_{cat} = 2765\ s^{-1}$), a K_m for H_4MPT of 0.6 mM, and a K_m for formyl-MFR of 0.5 mM were determined.

The findings indicate that the formyltransferases from *M. barkeri* and from *M. thermoautotrophicum* are very similar enzymes.

3.2. *Spectroscopic analysis of the products formed*

H_4MPT, N^5-formyl-H_4MPT and N^{10}-formyl-H_4MPT exhibit characterisitic UV spectra [1,5]. The H_4MPT spectrum shows maxima at 302 nm ($\epsilon = 15.2\ mM^{-1} \cdot cm^{-1}$) and 247 nm ($\epsilon = 22.5\ mM^{-1} \cdot cm^{-1}$) and a minimum at 276 nm ($\epsilon = 9.9\ mM^{-1} \cdot cm^{-1}$). The spectrum of N^5-formyl-H_4MPT has a maximum at 282 nm ($\epsilon = 16.6\ mM^{-1} \cdot cm^{-1}$) [6] and that of N^{10}-formyl-H_4MPT a maximum at 288 nm ($\epsilon = 11.6\ mM^{-1} \cdot cm^{-1}$). For the interpretation of the spectroscopic data it is of importance to note that at 282 nm only the N^5-isomer rather than the N^{10}-isomer shows a higher absorbance than H_4MPT. $\Delta\epsilon_{282}$ is 5.1 $mM^{-1} \cdot cm^{-1}$ for the

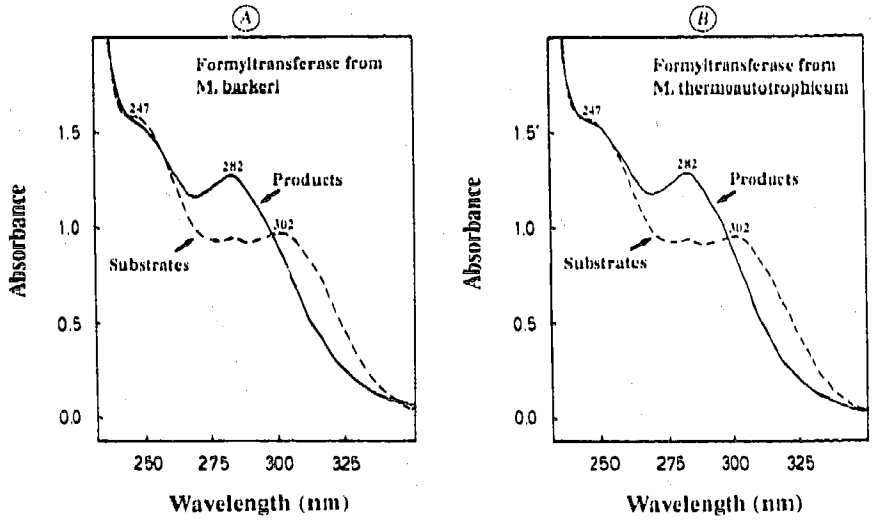

Fig. 2. UV spectra of substrates and products of the formyltransferase reaction catalyzed by purified enzyme (A) from *M. barkeri* and (B) from *M. thermoautotrophicum*. The dashed line represents the UV spectrum of the substrates before start of reaction with enzyme (0.13 mM H_4MPT and 0.18 mM formylmethanofuran). The solid line represents the UV spectrum of the products after completion of the reaction. The 0.7 ml assays were performed in 0.5 cm anaerobic cuvettes in 20 mM potassium phosphate pH 7.0 and at a temperature of 37°C. The experiment with the enzyme of *M. thermoautotrophicum* was also performed at 37°C rather than at 65°C since formyl-H_4MPT at high temperatures is unstable and is slowly converted to N^5,N^{10}-methenyl-H_4MPT.

H_4MPT/N^5-formyl-H_4MPT couple [6] and essentially 0 $mM^{-1} \cdot cm^{-1}$ for the H_4MPT/N^{10}-formyl-H_4 MPT couple. It is also of importance that formyl-MFR and MFR exhibit identical spectra with a maximum at 273 nm ($\epsilon = 1.34$ $mM^{-1} \cdot cm^{-1}$) and a shoulder at 277 nm ($\epsilon = 1.14$ $mM^{-1} \cdot cm^{-1}$) [1,9]. (Note that in the 280 nm region the ϵ values of MFR and formyl-MFR are by an order of magnitude lower than those of tetrahydromethanopterin and its derivatives.) From the spectra of the individual compounds it can therefore be predicted that only the formation of N^5-formyl-H_4MPT from H_4MPT and formyl-MFR is associated with an increase in absorbance at 282 nm and that the formation of the N^{10}-isomer is not.

The UV spectrum of an assay solution containing 0.13 mM H_4MPT and 0.18 mM formyl-MFR before start of the formyltransferase reaction is shown in Fig. 2A and B (dashed lines). It is composed of the spectra of H_4MPT and of formyl-MFR.

The UV spectrum of the products formed from H_4MPT and formyl-MFR in the presence of purified formyltransferase from *M. barkeri* is shown in Fig. 2A (solid line). An increase in absorbance at 282 nm was observed indicating that H_4MPT was converted to N_5-formyl-H_4MPT rather than to N^{10}-formyl-H_4MPT. The increase in absorbance was that calculated for an over 90% conversion to the N^5-isomer using a $\Delta\epsilon_{282}$ of 5.1 $mM^{-1} \cdot cm^{-1}$. When the spectrum of a 0.18 mM MFR solution was substracted from the product spectrum exactly that published for pure N_5-formyl-H_4MPT was obtained (difference spectrum not shown).

In Fig. 2B the UV spectrum of the products formed from H_4MPT (0.13 mM) and formyl-MFR (0.18 mM) in the presence of purified formyltransferase from *M. thermoautotrophicum* (strain Marburg) is shown. It is indistinguishable from that obtained in the parallel experiment with purified formyltransferase from *M. barkeri*. Formyltransferase from *M. thermoautotrophicum* has been shown to yield N^5-formyl-H_4MPT [6].

The substrate and product spectra (dashed and solid lines, respectively) intersected at 296 nm. When the progress of the formyltransferase reaction was followed by serial scanning a clearly defined isobestic point at 296 nm was obtained (data not shown) indicating that only one *N*-formyl-H_4MPT isomer was formed from H_4MPT and formyl-MFR [6].

3.3. *O_2-stability of the product formed*

N^5-Formyl-H_4MPT is stable in air whereas the N^{10}-isomer and H_4MPT are not [1,10]. We therefore tested whether the UV spectrum of the product formed from H_4MPT (0.13 mM) and formyl-MFR (0.18 mM) in the presence of formyltransferase from *M. barkeri* was affected by O_2. No change in spectrum was observed when, after completion of the reaction, the assay solution was equilibrated with air. When, as a control, an N^{10}-formyl-H_4MPT solution was exposed to air under the same conditions the UV spectrum characteristically changed to one with maxima at 277 nm and 320 nm [10]. These findings indicate that N^{10}-formyl-H_4MPT was not the product formed in the formyltransferase reaction.

3.4. *Conversion of the product to N^5,N^{10}-methenyl-H_4MPT in the presence of cyclohydrolase from* M. thermoautotrophicum

The N^5,N^{10}-methenyl-H_4MPT cyclohydrolase of *M. thermoautotrophicum* is specific for N^5-formyl-H_4MPT as substrate [7]. We therefore tested whether the product formed by purified formyltransferase from *M. barkeri* is converted to N^5,N^{10}-methenyl-H_4MPT in the presence of purified cyclohydrolase from *M. thermoautotrophicum*. Indeed it was found that after addition of the latter enzyme the spectrum of the product rapidly changed to that of N^5,N^{10}-methenyl-H_4MPT with absorption maxima at 335 nm ($\epsilon = 21.6$ $mM^{-1} \cdot cm^{-1}$) and at 287 nm ($\epsilon = 13.3$ $mM^{-1} \cdot cm^{-1}$) and a minimum at 261 nm ($\epsilon = 7.4$ $mM^{-1} \cdot cm^{-1}$). At pH 6.2 an almost complete conversion was observed and the rate of conversion corresponded to the cyclohydrolase activity added. This finding indicates that the N^5-isomer is formed in the formyltransferase reaction.

3.5. *The formyl-H_4MPT isomer used by cyclohydrolase of* M. barkeri

It was tested whether cell extracts of *M. barkeri* can catalyze the conversion of N^{10}-formyl-H_4MPT (0.05 mM) to N^5,N^{10}-methenyl-H_4MPT, the generation of which was followed at 335 nm ($\epsilon = 21.6$ $mM^{-1} \cdot cm^{-1}$) (in 100 mM Mes/KOH pH 6.2). The result was negative. When to the assay N^5-formyl-H_4MPT (0.07 mM), or H_4MPT (0.07 mM) plus formyl-MFR (0.1 mM) were added as an internal control a rapid formation of N^5,N^{10}-methenyl-H_4MPT was observed. The specific rate of conversion was approximately 1.6 U/mg protein at pH 6.2. These findings indicate that the cyclohydrolase of *M. barkeri* only catalyzes the conversion of the N^5- rather than of the N^{10}-isomer.

3.6. *Conclusion*

It was shown that both the formyltransferase and the cyclohydrolase from methanol grown *M. barkeri* are specific for N^5-formyl-H_4MPT. Both enzymes were present in cell extracts in catabolic activities (>1 U/mg). It is therefore concluded that methanogenesis from methanol in *M. barkeri* involves N^5-formyl-H_4MPT as intermediate as this has been reported for methanogenesis from H_2 and CO_2 in *M. thermoautotrophicum*.

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