N-Furfurylformamide as a pseudo-substrate for formylmethanofuran converting enzymes from methanogenic bacteria

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Methanofuran (4-[*N*-(4,5,7-tricarboxyheptanoyl-γ-L-glutamyl)-γ-L-glutamyl)-*p*-(β-aminoethyl)phenoxymethyl]-2-(aminomethyl)furan is a coenzyme involved in methanogenesis. The *N*-formyl derivative is an intermediate in the reduction of CO_2 to CH_4 and the disproportionation of methanol to CO_2 and CH_4. Formylmethanofuran dehydrogenase and formylmethanofuran:tetrahydromethanopterin formyltransferase are the enzymes catalyzing its conversions. We report here that the two enzymes from *Methanosarcina barkeri* and the formyltransferase from *Methanobacterium thermoautotrophicum* can also use *N*-furfurylformamide as a pseudo-substrate albeit with higher apparent K_m and lower apparent V_{max} values. *N*-Methylformamide, formamide, and formate were not converted indicating that the furfurylamine moiety of methanofuran is the minimum structure required for the correct binding of the coenzyme.

Methanofuran; Formylmethanofuran; Methanogenesis; *Methanosarcina barkeri*; *Methanobacterium thermoautotrophicum*; Coenzyme specificity

1. INTRODUCTION

The reduction of CO_2 to methane and the oxidation of methanol to CO_2 in ethanogenic archaebacteria proceed via carrier bound C_1-units [1]. One of the carriers involved is methanofuran (MFR) [2] (Fig. 1). Formylmethanofuran (CHO-MFR) [3] (Fig. 1) is the intermediate in the conversion of CO_2 plus tetrahydromethanopterin (H_4MPT) to N^5-formyltetrahydromethanopterin (CHO-H_4MPT) via reactions (1) and (2):

$$CO_2 + MFR + 2[H] \rightleftharpoons CHO\text{-}MFR + H_2O \quad (1)$$
$$E^{\circ\prime} = -500\ mV$$
$$CHO\text{-}MFR + H_4MPT \rightleftharpoons CHO\text{-}H_4MRP + MFR \quad (2)$$
$$\Delta G^{\circ\prime} = -4.4\ kJ/mol$$

Reaction a is catalyzed by the molybdoenzyme formylmethanofuran dehydrogenase [4-6] and reaction b by formylmethanofuran:tetrahydromethanopterin formyltransferase [7,8].

Methanofuran isolated from *Methanobacterium thermoautotrophicum* has the structure depicted in Fig. 1. The coenzyme from *Methanosarcina barkeri* differs in that the terminal C_{10}-tetracarboxylic acid is replaced by two glutamates [9,10]. Despite this difference the methanofuran isolated from *M. thermoautotrophicum* is active with the enzymes from *M. barkeri* and vice versa. It was therefore of interest to determine the significance of the side chain (R) of methanofuran (Fig. 1) for the recognition of this coenzyme by formylmethanofuran converting enzymes.

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2. MATERIALS AND METHODS

Methanosarcina barkeri (strain Fusaro, DSM 804) and *Methanobacterium thermoautotrophicum* (strain Marburg, DSM 2133) were from the Deutsche Sammlung von Mikroorganismen (Braunschweig, FRG). The cells were grown, harvested, and cell extracts prepared as described before [11]. Formylmethanofuran:tetrahydromethanopterin formyltransferase and formylmethanofuran dehydrogenase were purified via published procedures [5,7]. Assay conditions for these enzymes are given in the legends to the tables and figures.

Methanofuran and tetrahydromethanopterin were isolated from *M. thermoautotrophicum* [12]. Formylmethanofuran was synthesized from methanofuran and 4-nitrophenylformate (Fluka Chemie AG, Buchs, Switzerland) [7].

N-Furfurylformamide was prepared from furfurylamine (Merck AG, Darmstadt, FRG): a solution of 17.9 g (17.0 ml, 184 mmol) furfurylamine and 190 mg (1.00 mmol) *p*-toluenesulphonic acid monohydrate in 170 ml absolute ethyl formate was refluxed under argon for 48 h. The solvent was removed at ca. 20°C, 15 Torr ()FG(2000 Pa), and the residual yellow oil was passed twice through silica gel (2 × 100 g, eluting with ether). The resulting pale yellow oil was finally purified by Kugelrohr distillation at 120°C, 0.01 Torr ()FG(1.3 Pa), affording 18.9 g (82%) *N*-furfurylformamide as a colorless oil. Elemental analysis: $C_6H_7NO_2$ (125.13). Calculated: C, 57.59; H, 5.64; N, 11.19. Found: C, 57.44; H, 5.75; N, 11.14. ^{1}H NMR (270 MHZ, d_6-DMSO): δ/ppm = 4.30 (dt, J = 5.8 Hz, J = 0.8 Hz; 2H, -CH_2-NH-), 6.24-6.28 (m; 1H, 3-H), 6.39 (dd, J = 3.1 Hz, J = 1.9 Hz; 1H, 4-H), 7.58 (dd, J = 1.9 Hz, J = 0.8 Hz; 1H, 5-H), 8.08 (mc; 1H, -CHO), 8.48 (br s; 1H, NH). The resonances at 6.24-6.28, 6.39, 7.58 and 8.08 ppm were accompanied by a second set of signals of ca. 12% intensity and shifted to lower field by 0.01-0.1 ppm. The observation of two sets of NMR-signals in a ratio of ap-

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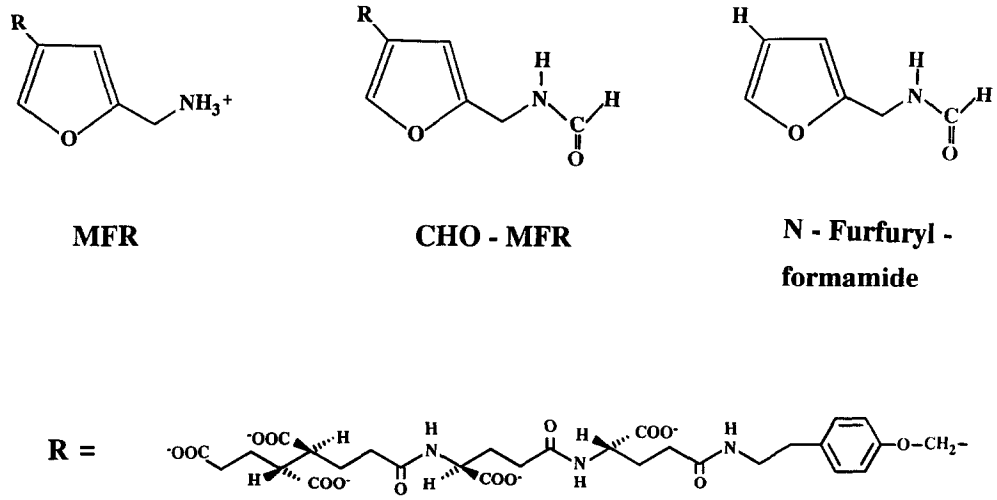

Fig. 1. Structure of methanofuran (MFR), of formylmethanofuran (CHO-MFR), and of *N*-furfurylformamide.

proximately 9.1 is a common phenomenon for *N*-mono-substituted formamides. The effect is due to hindered rotation around the amide C-N bond. The major conformer can be assumed to have Z-configuration around the amide C-N bond (as is the case for *N*-methylformamide). Infrared spectroscopy (film): γ = 3293, 3049, 2872, 1694, 1666, 1531, 1506, 1385, 1346, 1233, 1197, 1148, 1078, 1015 cm^{-1}. Ultraviolet spectroscopy (methanol): λ_{max} (1gϵ) = 214 nm (4.011).

3. RESULTS

The substrate specificity of formylmethanofuran:tetrahydromethanopterin formyltransferase and of formylmethanofuran dehydrogenase from methanol grown *M. barkeri* and from H_2/CO_2 grown *M. thermoautotrophicum* was analyzed. The experiments were performed with cell extracts and with purified enzyme preparations. Compounds tested were formylmethanofuran, *N*-furfurylformamide, *N*-methylformamide, formamide, and formate. In the assays for formyltransferase activity a constant concentration of tetrahydromethanopterin of 0.065 mM was chosen.

3.1. *Formyltransferase from* M. barkeri

Cell extracts of *M. barkeri* were found to contain beside formylmethanofuran:tetrahydromethanopterin formyltransferase high activities of methenyltetrahydromethanopterin cyclohydrolase ($\approx$2 μmol/min/mg protein). The latter enzyme catalyzes the conversion of formyltetrahydromethanopterin (CHO-H_4MPT) to N^5,N^{10}-methenyltetrahydromethanopterin (CH$\equiv H_4$MPT) [13,14].

$$\text{CHO-H}_4\text{MPT} + \text{H}^+ \rightleftharpoons \text{CH}\equiv\text{H}_4\text{MPT}^+ + \text{H}_2\text{O} \quad (3)$$
$$\Delta G^{\circ\prime} = -4.6 \text{ kJ/mol}$$

Due to the presence of both formyltransferase and cyclohydrolase CH$\equiv H_4$MPT was formed when the extracts were incubated with formylmethanofuran and tetrahydromethanopterin. The formation of CH$\equiv H_4$MPT, which was followed photometrically at 340 nm, began without any lag period indicating that the cyclohydrolase reaction was not rate limiting. The initial rate was therefore taken as a measure of formyltransferase activity.

With this assay system it was found that formyltransferase from *M. barkeri* can also use *N*-furfurylformamide as substrate albeit with a much higher apparent K_m and a lower apparent V_{max}. The concentration dependence of the rate followed Michaelis-Menten kinetics. From reciprocal plots an apparent K_m for formylmethanofuran of 0.06 mM and an apparent V_{max} of 1.8 μmol/min/mg protein were obtained. The respective values of *N*-furfurylformamide were 55 mM and 0.025 μmol/min/mg (Fig. 2). The cell extracts showed no activity with N-methylformamide (200 mM), formamide (200 mM), or formate (200 mM).

The substrate specificity was also tested with a 500-fold purified formyltransferase from *M. barkeri*. The preparation was free of cyclohydrolase activity. Therefore, the formation of N^5-formyltetrahydromethanopterin could be followed directly by measuring the increase of absorbance at 282 nm (ϵ_{282} = 5.1 $mM^{+1} \cdot cm^{-1}$) [7]. The purified enzyme was also found to use *N*-furfurylformamide but not *N*-methylformamide, formamide, or formate as substrates. The specific activity with *N*-furfurylformamide parallelled that with formylmethanofuran indicating that the conversion of the pseudo-substrate and of the natural substrate was catalyzed by the same enzyme.

3.2. *Formylmethanofuran dehydrogenase from M. barkeri*

Formylmethanofuran dehydrogenase was tested by measuring the reduction of methylviologen (5 mM) with formylmethanofuran. Cell extracts of *M. barkeri* catalyzed the reaction with an apparent V_{max} of 1.6 μmol/min/mg protein. The apparent K_m for for-

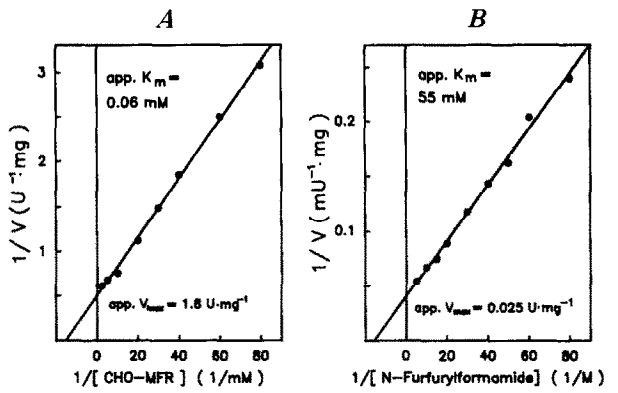

Fig. 2. Activity of formylmethanofuran: tetrahydromethanopterin formyltransferase from *M. barkeri* (A) with formylmethanofuran as substrate and (B) with *N*-furfurylformamide as pseudo-substrate. The assays were performed in 1.5 ml anaerobic cuvettes containing 0.7 ml of the following mixture: 100 mM Mes buffer, pH 7; 65 μM tetrahydromethanopterin; formylmethanofuran or *N*-furfurylformamide in concentrations indicated; and 15 μg (A) or 1.2 mg (B) cell extract protein. The gas phase was N_2 and the cuvette was equilibrated to 37°C. The reaction was started by the addition of enzyme and was monitored by recording the absorbance increase at 340 nm due to the formation of CH)≡(H₄MPT (ϵ_{340} = 20 $mM^{-1} \cdot cm^{-1}$). One unit = 1 μmol CH≡H₄MPT formed per min. The data were plotted according to Lineweaver-Burk.

mylmethanofuran was 0.01 mM. The cell extract was also found to mediate the reduction of methylviologen with *N*-furfurylformamide. The apparent V_{max} and apparent K_m were 0.2 μmol/min/mg and 200 mM, respectively. No activity was observed with *N*-methylformamide (200 mM), formamide (200 mM), and formate (200 mM). Purified formylmethanofuran dehydrogenase from *M. barkeri* showed the same substrate specificity as indicated by a comparison of the apparent K_m and V_{max} values summarized in Table I.

Table I

Activity of formylmethanofuran dehydrogenase from *M. barkeri* with formylmethanofuran as substrate and with *N*-furfurylformamide as pseudo-substrate

Fraction	app. V_{max} (u/mg)	app. K_m (mM)
Cell extract		
Formylmethanofuran	1.6	0.01
N-furfurylformamide	0.2	200
Purified		enzyme
Formylmethanofuran	175	0.02
N-Furfurylformamide	20	200

The assays were performed in 1 ml anaerobic cuvettes containing 0.3 ml of the following mixtures: 50 mM Tricine buffer pH 8; 5 mM methylviologen; 0.1 mM formylmethanofuran or 800 mM *N*-furfurylformamide; and 200–500 μg cell extract protein or 1–3 μg purified enzyme protein. The gas phase was N_2 and the mixture was equilibrated to 37°C. The reaction was started by the addition of enzyme and followed by recording the absorbance increase at 578 nm due to the formation of reduced methylviologen (ϵ_{578} = 9.7 $mM^{-1} \cdot cm^{-1}$). 1 U = 2 μM methylviologen reduced per minute.

3.3. *Enzymes from* M. thermoautotrophicum

3.3.1. Formyltransferase

The enzyme from *M. thermoautotrophicum* was found to use *N*-furfurylformamide as substrate. In cell extracts (pH 6.2, 60°C) the apparent V_{max} was 0.07 μmol/min/mg and the apparent K_m was 70 mM as compared to an apparent V_{max} of 2 μmol/min/mg [7] and an apparent K_m of 0.06 mM for the natural substrate.

3.2.2. Formylmethanofuran dehydrogenase

Surprisingly, the enzyme from *M. thermoautotrophicum* showed no activity with *N*-furfurylformamide. The latter compound also did not inhibit methylviologen reduction with formylmethanofuran, which proceeded at a specific activity of 2 μmol/min/mg (pH 8, 65°C) [4]. These findings were confirmed with partially purified enzyme from *M. thermoautotrophicum*. Thus formylmethanofuran dehydrogenase from *M. thermoautotrophicum* and from *M. barkeri* differ markedly with respect to substrate specificity.

4. DISCUSSION

Formylmethanofuran:tetrahydromethanopterin formyltransferase from *M. barkeri* and from *M. thermoautotrophicum* and formylmethanofuran dehydrogenase from *M. barkeri* were found to use *N*-furfurylformamide as a pseudo-substrate. *N*-Methylformamide, formamide, or formate were not used by these enzymes. This finding indicates that the furfurylamine moiety of methanofuran is the minimum structure required for the correct binding of this coenzyme. The formyltransferase and the formylmethanofuran dehydrogenase, therefore, must have a recognition site for the furfurylamine structure.

The enzymes investigated showed a much higher apparent K_m and a much lower apparent V_{max} for *N*-furfurylformamide than for the natural substrate. The side chain (R) in methanofuran (Fig. 1) is therefore also of considerable importance for the kinetics and energetics of coenzyme binding. In the case of formylmethanofuran dehydrogenase from *M. thermoautotrophicum* the presence of the side chain is even a prerequisite for the binding. *N*-Furfurylformamide was neither a substrate nor a competitive inhibitor for this enzyme.

The finding that only the moiety with the functional group is required for coenzyme activity is not without a precedent. For instance, coenzyme A-dependent enzymes are known that can use *N*-acetylcysteamine as pseudo-substrate [15]. β-Ketothiolase from sheep liver is such an enzyme. Other coenzyme A-dependent enzymes are more specific. Phosphotransacetylase from *Clostridium kluyveri* belongs to the latter group. This enzyme does not even accept dephospho-CoA as substrate [16].

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