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

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Methanofuran (4-[N-(4,5,7-tricarboxyheptanoyl-4-L-glutamyl)-4-L-glutamyl]-p-(8-aminoethyl)phenoxymethyl]-2-(aminomethyl)furan is a coenzyme involved in methanogenesis. The N-formyl derivative is an intermediate in the reduction of CO₂ to CH₄ and the disproportionation of methanol to CO₂ and CH₄. Formylmethanofuran dehydrogenase and formylmethanofuran:tetrahydromethanopterin formyltransferase are the enzymes catalyzing its conversions. We report here that the two enzymes from Methanobacterium thermoautotrophicum can also use N-furfurylformamide as a pseudo-substrate albeit with higher apparent Kₘ and lower apparent Vₘₐₚ values.

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

1. INTRODUCTION

The reduction of CO₂ to methane and the oxidation of methanol to CO₂ in ethanogenic archaebacteria proceed via carrier bound C₁-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 CO₂ plus tetrahydromethanopterin (H₄MPT) to N₂-formyltetrahydromethanopterin (CHO-H₄MPT) via reactions (1) and (2):

\[
\text{CO}_2 + \text{MFR} + 2[\text{H}] \rightarrow \text{CHO-MFR} + \text{H}_2\text{O} \quad (1)
\]

\[
\Delta G^{\circ} = -500 \text{ mV}
\]

\[
\text{CHO-MFR} + \text{H}_4\text{MPT} \rightarrow \text{CHO-H}_4\text{MRP} + \text{MFR} \quad (2)
\]

\[
\Delta G^{\circ} = -4.4 \text{ kJ/mol}
\]

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 C₁₀-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.

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, Buch, 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-toluenesulfonic 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 (1 Torr = 133 Pa), and the residual yellow oil was passed twice through silica gel (2 × 100 g, eutect with ether). The resulting pale yellow oil was finally purified by Kugelrohr distillation at 120°C, 0.01 Torr (1 Torr = 133 Pa) affording 18.9 g (82%) N-furfurylformamide.

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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): $\gamma = 3293, 3049, 2877, 1694, 1531, 1506, 1385, 1346, 1233, 1197, 1148, 1078, 1015 \text{ cm}^{-1}$. Ultraviolet spectroscopy (methanol): $\lambda_{\text{max}} (\text{lg}) = 214 \text{ nm}$.

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 ($\sim 2 \mu\text{mol/min/mg}$ protein). The latter enzyme catalyzes the conversion of formyltetrahydromethanopterin (CHO-H$_4$MPT) to $N^5,N^{10}$-methenyltetrahydromethanopterin (CH$=\text{H}_4$MPT) [13,14].

$$\text{CHO-H}_4\text{MPT} + \text{H}^+ \rightleftharpoons \text{CH}=\text{H}_4\text{MPT}^+ + \text{H}_2\text{O}(3)$$

$\Delta G^\circ = -4.6 \text{ kJ/mol}$

Due to the presence of both formyltransferase and cyclohydrolase CH$=\text{H}_4$MPT was formed when the extracts were incubated with formylmethanofuran and tetrahydromethanopterin. The formation of CH$=\text{H}_4$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 $V_{\text{max}}$. 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 $V_{\text{max}}$ of 1.8 $\mu$mol/min/mg protein were obtained.

The respective values of N-furfurylformamide were 55 mM and 0.025 $\mu$mol/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 ($\varepsilon_{282} = 5.1 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) [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 paralleled 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 $V_{\text{max}}$ of 1.6 $\mu$mol/min/mg protein. The apparent $K_m$ for for-
Formylmethylfuran was 0.01 mM. The cell extract was also found to mediate the reduction of methylviologen with N-furfurylformamide. The apparent V\textsubscript{max} and apparent K\textsubscript{m} were 0.2 \mu mol/min/mg and 200 mM, respectively. No activity was observed with N-methylfor-...

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### Table 1

<table>
<thead>
<tr>
<th>Fraction</th>
<th>app. V\textsubscript{max} (u/mg)</th>
<th>app. K\textsubscript{m} (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formylmethanofuran</td>
<td>1.6</td>
<td>0.01</td>
</tr>
<tr>
<td>N-Furfurylformamide</td>
<td>0.2</td>
<td>200</td>
</tr>
<tr>
<td>Purified enzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formylmethanofuran</td>
<td>175</td>
<td>0.02</td>
</tr>
<tr>
<td>N-Furfurylformamide</td>
<td>20</td>
<td>200</td>
</tr>
</tbody>
</table>

The assays were performed in 1 ml anaerobic cuvettes containing 0.3 ml of the following mixtures: 50 mM Tricine buffer pH 7; 65 \mu M tetrahydromethanopterin; formylmethanofuran or N-furfurylformamide in concentrations indicated; and 15 \mu g (A) or 1.2 mg (B) cell extract protein. The gas phase was N\textsubscript{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 reduced methylviologen (\epsilon = 9.7 M\textsuperscript{-1} cm\textsuperscript{-1}). One unit = 1 \mu mol CH=HMP Fi per min.

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\textsubscript{max} was 0.07 \mu mol/min/mg and the apparent K\textsubscript{m} was 70 mM as compared to an apparent V\textsubscript{max} of 2 \mu mol/min/mg [7] and an apparent K\textsubscript{m} of 0.06 mM for the natural substrate.

3.3.2. 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 \mu mol/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 furfurylamino 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\textsubscript{m} and a much lower apparent V\textsubscript{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-acetyllysysteamine as pseudo-substrate [15]. \beta-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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REFERENCES
