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Formylmethanofuran dehydrogenase activity in cell extracts of *Methanobacterium thermoautotrophicum* and of *Methanosarcina barkeri*

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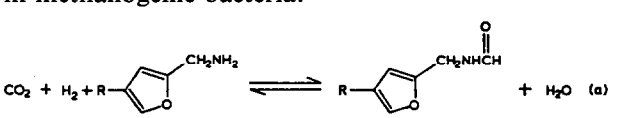
Cell extracts of *Methanobacterium thermoautotrophicum* catalyzed the reduction of methyl viologen (apparent $K_m = 0.1$ mM) with formylmethanofuran (apparent $K_m = 10$ μM) at a specific rate of 4 μmol · min^{-1} · mg protein^{-1}. Coenzyme F_{420} (apparent $K_m = 25$ μM) rather than NAD, NADP, FAD, or FMN was used as physiological electron acceptor. With coenzyme F_{420} the specific activity was 0.4 μmol · min^{-1} · mg^{-1}. More than 60% of the formylmethanofuran dehydrogenase activity was associated with the pellet fraction, which was obtained by centrifugation at 160000 × *g*. An enzyme system with very similar properties was also found to be present in cell extracts of *Methanosarcina barkeri* grown on methanol. In both organisms the formylmethanofuran dehydrogenase activity was rapidly inactivated by cyanide.

Methanofuran; Coenzyme F_{420}; Methanogenesis; Methanol oxidation; (*Methanobacterium, Methanosarcina*)

1. INTRODUCTION

The formation of formylmethanofuran (CHO-MFR) from CO_2, H_2, and methanofuran (MFR) is the initial redox step in CO_2 reduction to methane in methanogenic bacteria.



This has been demonstrated with cell extracts of *Methanobacterium thermoautotrophicum* [1-9] and of other methanogenic bacteria [10-12]. The free energy change ($\Delta G^{0\prime}$) associated with reaction a has been estimated to be 16 kJ/mol [13]. The reverse reaction is thus energetically more favourable. In accordance herewith it was found that in the absence of tetrahydromethanopterin cell extracts of *Mb. thermoautotrophicum* readily converted ^{14}CHO-MFR to $^{14}CO_2$ even under an H_2/CO_2 atmosphere (5).

In the natural habitats of methanogenic bacteria the H_2 partial pressure is generally only of the order of 1-10 Pa [14]. Under these conditions CO_2 reduction with H_2 to CHO-MFR is even more endergonic and can thus probably proceed only when coupled to an exergonic reaction. The mode of coupling is still not known. Available evidence suggests that ATP is not directly involved [15] and that reaction a could be driven by the electrochemical H^+ or Na^+ potential (see [16,17]).

The enzyme system mediating reaction a has not yet been resolved. Most likely a hydrogenase and a formylmethanofuran dehydrogenase are involved, which are connected by an electron carrier or an electron-transport chain. We describe here, for the first time, some properties of the formylmethanofuran dehydrogenase activity in two representative methanogenic bacteria.

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2. MATERIALS AND METHODS

Methyl viologen was from Serva (Heidelberg) and Tricine (*N*-[Tris(hydroxymethyl)methyl]glycine) from Merck (Darmstadt).

Methanobacterium thermoautotrophicum (strain Marburg) (DSM 2133) and *Methanosarcina barkeri* (strain Fusaro) (DSM 804) were from the Deutsche Sammlung von Mikroorganismen (Braunschweig). The cells were grown, harvested, and stored as described [17–19]. Cell suspensions of *Mb. thermoautotrophicum* were prepared in 50 mM Tris-HCl buffer (pH 7) containing 2 mM dithiothreitol and 30 mM $MgCl_2$ at a concentration of 50 mg protein/ml. Cell suspensions of *M. barkeri* were prepared in 50 mM potassium phosphate buffer (pH 7) containing 0.5 mM dithiothreitol, 2 mM $MgCl_2$, and 50 mM NaCl. The suspensions were passed twice through a French pressure cell at 1100×10^5 Pa. Cell debris and unbroken cells were removed by centrifugation for 30 min at $27\,000 \times g$. The $27\,000 \times g$ supernatant is referred to as cell extract.

Enzyme activities were determined in 1.3 ml anaerobic cuvettes closed with rubber stoppers. The cuvettes contained 0.7 ml assay mixture (composition listed in table 1) and N_2 in the gas phase at 1.2×10^5 Pa. The reduction of methyl viologen ($\epsilon_{578} = 9700$ $cm^{-1} \cdot M^{-1}$) and of coenzyme F_{420} ($\epsilon_{420} = 39\,000$ $cm^{-1} \cdot M^{-1}$ at pH 7; $\Delta\epsilon_{420}$ (ox minus red) = 35 100 $cm^{-1} \cdot M^{-1}$ at pH 7) was followed photometrically at 578 and 420 nm, respectively. All additions were made by syringes.

Methanofuran (MFR), tetrahydromethanopterin (H_4MPT), and coenzyme F_{420} were isolated from *Mb. thermoautotrophicum* [20]. Formylmethanofuran (CHO-MFR) was prepared as described in [6]. The concentration of methanofuran solutions was determined with ninhydrin using furfurylamine as standard. ϵ_{274} was found to be 1500 $cm^{-1} \cdot M^{-1}$.

3. RESULTS

In a previous work [7] the enzyme activity mediating the reversible reduction of CO_2 to CHO-MFR was tested by measuring the formation of CHO-MFR, which was quantified after separation from MFR by HPLC. This is a tedious procedure which does not allow detailed kinetic analysis. Therefore, a continuous photometric assay was sought. It was found that cell extracts of *Mb. thermoautotrophicum* and of *M. barkeri* catalyzed the reduction of methyl viologen and of coenzyme F_{420} with CHO-MFR. Methyl viologen is a 1-electron redox dye with an $E^{\circ\prime} = -446$ mV. Coenzyme F_{420} is a 5′-deazaflavin found in all methanogenic bacteria. It is a two-electron (hydride) carrier with an $E^{\circ\prime} = -360$ mV [21,22]. NAD, NADP, or flavins were not reduced by CHO-MFR.

3.1. *Methyl viologen reduction with CHO-MFR*

Cell extracts of *Mb. thermoautotrophicum* mediated the reduction of methyl viologen (5 mM) with CHO-MFR (0.1 mM) rather than with formate (5 mM). The rate increased linearly with protein in the concentration range tested (1–100 μg per ml). The specific rate was 4 μmol methyl viologen reduced $min^{-1} \cdot mg^{-1}$ = 2 μmol CHO-MFR oxidized $min^{-1} \cdot mg^{-1}$. 1.8–1.9 mol reduced methyl viologen was formed per mol CHO-MFR added (fig.1). The rate dependence on the substrate concentration followed Michaelis-Menten kinetics. From reciprocal plots of $1/v$ vs $1/[S]$ apparent K_m values for CHO-MFR and methyl viologen of 10 μM and 0.1 mM, respectively, were obtained. The pH optimum in Tricine-NaOH buffer was near 8 (table 1).

More than 60% of the activity catalyzing methyl viologen reduction with CHO-MFR was associated with the pellet fraction obtained by centrifugation at $160\,000 \times g$ for 120 min, 30–40% being found in the supernatant. The distribution of three other enzymes was determined as a control. Methylene-H_4MPT dehydrogenase ($M_r \sim 50\,000$) [23] and methyl coenzyme M reductase ($M_r = 300\,000$) [18] were almost quantitatively recovered in the supernatant fraction. The distribution of the F_{420}-reducing hydrogenase ($M_r \sim 800\,000$) [24] was 50% in the pellet and 50% in the supernatant fraction.

3.2. *Coenzyme F_{420} reduction with CHO-MFR*

In cell extracts of *Mb. thermoautotrophicum* the specific rate of reduction of coenzyme F_{420} (50 μM) with CHO-MFR (0.1 mM) was 0.4 μmol $\cdot$ $min^{-1} \cdot mg^{-1}$. Up to 70% of the activity was associated with the $160\,000 \times g$ pellet and only 20–30% with the supernatant fraction. The dependence of the rate on the protein concentration was linear when the pellet fraction was tested; it was parabolic in the case of the supernatant fraction when low protein concentrations were employed.

The apparent K_m values for coenzyme F_{420} and for CHO-MFR were determined with the pellet fractions and found to be 25 and 10 μM, respectively (table 1). The pH optimum in potassium/phosphate buffer was 7.

3.3. *Inactivation by cyanide*

Cyanide was found to inactivate rapidly the enzyme system mediating the reduction of methyl

Table 1

Kinetic properties of the CHO-MFR dehydrogenase activity present in cell extracts of *Mb. thermoautotrophicum* and of *M. barkeri*

	CHO-MFR dehydrogenase activity	
	Methanobacterium thermoautotrophicum (65°C)	*Methanosarcina barkeri* (37°C)
Specific activity		
With methyl viologen[a]	2 U/mg	1.2 U/mg
With coenzyme F_{420}[b]	0.4 U/mg	0.06 U/mg
Apparent K_m values		
For CHO-MFR[c]	10 μM	10 μM
For coenzyme F_{420}[d]	25 μM	25 μM
For methyl viologen[e]	0.1 mM	1 mM
pH optimum		
With methyl viologen	8	7.5
With coenzyme F_{420}	7	7

[a] 50 mM Tricine-NaOH buffer (pH 8); 2 mM dithiothreitol; 50 mM NaCl; 0.1 mM CHO-MFR; 5 mM methyl viologen; 40–200 μg cell extract protein
[b] 50 mM potassium phosphate buffer (pH 7); 2 mM dithiothreitol; 50 mM NaCl; 0.1 mM CHO-MFR; 40 μM coenzyme F_{420}; 0.1–0.5 mg cell extract protein
[c] With 5 mM methyl viologen as electron acceptor at pH 8
[d] With 0.1 mM CHO-MFR at pH 7
[e] With 0.1 mM CHO-MFR at pH 8
1 U = 1 μmol F_{420} or 2 μmol methyl viologen reduced per min

viologen and of coenzyme F_{420} with CHO-MFR. At 0.1 mM cyanide approx. 50% of the activity was lost within 3 min (fig.2). Azide (10 mM) had no significant effect on CHO-MFR dehydrogenase activity in *Mb. thermoautotrophicum*.

3.4. *CHO-MFR dehydrogenase activity in cell extracts of* M. barkeri.

Cell extracts of *M. barkeri* mediated the reduction of methyl viologen and of coenzyme F_{420} with CHO-MFR rather than with formate. After cen-

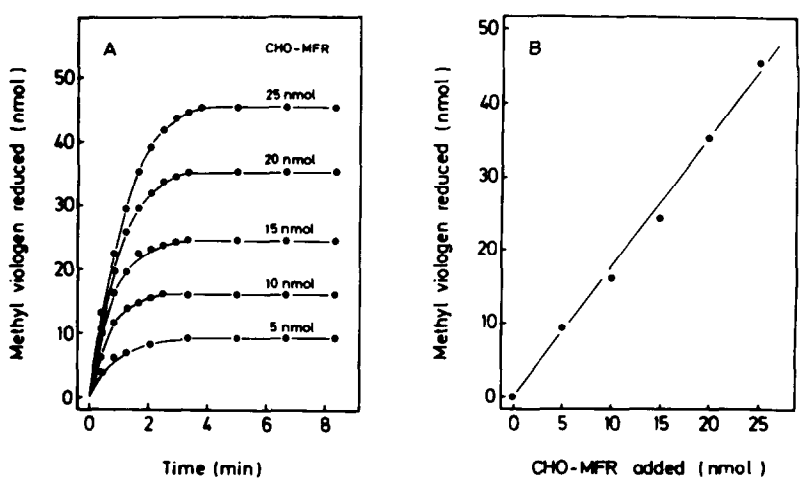

Fig.1. Reduction of methyl viologen with CHO-MFR in cell extracts of *Mb. thermoautotrophicum*. (A) Kinetics and (B) stoichiometry of the reaction. The 0.7 ml assay mixtures contained: 50 mM Tricine-NaOH buffer (pH 8); 2 mM dithiothreitol; 5 mM methyl viologen; 50 mM NaCl; and the amounts of CHO-MFR as indicated. The reactions were started by the addition of 5 μg pellet fraction protein.

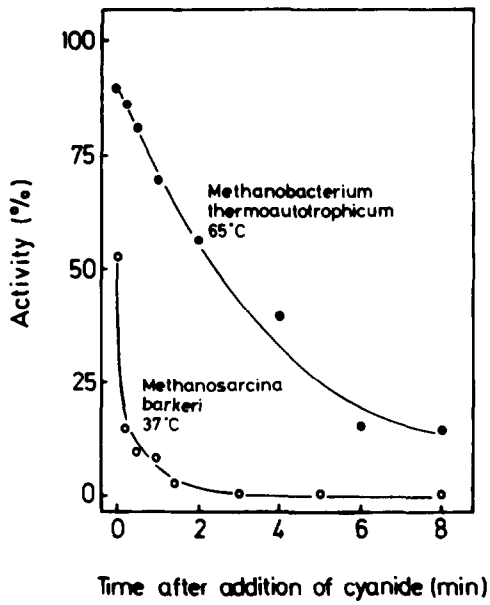

Fig.2. Inactivation by cyanide of the CHO-MFR dehydrogenase activity in cell extracts of *Mb. thermoautotrophicum* and of *M. barkeri*. The experiment was performed in 1.3-ml anaerobic cuvettes containing 0.7 ml mixture of the following composition: 50 mM Tricine-NaOH buffer (pH 8); 2 mM dithiothreitol; 50 mM NaCl; and 5 mM methyl viologen. The gas phase was N_2 at 1.2×10^5 Pa. After temperature equilibration to 65 or 37°C, respectively, 0.1 mg pellet fraction protein and 70 nmol KCN were successively added. After incubation to 0, 1, 2, 5, and 8 min the activity was tested by determining the initial rate of methyl viologen reduction with CHO-MFR (100 nmol) which was injected with a syringe. 100% activity refers to the rate in a cuvette not containing cyanide.

trifugation (120 min at $160\,000\times g$) approx. 50% of the activity was recovered in the pellet fraction. Specific activities and apparent K_m values were determined and are given in table 1.

The specific activity with methyl viologen was 1.2 $\mu mol\cdot min^{-1}\cdot mg^{-1}$. The specific rate with coenzyme F_{420} was only 0.06 $\mu mol\cdot min^{-1}\cdot mg^{-1}$ (table 1). The low specific activity may be due to the fact that coenzyme F_{420} from *Mb. thermoautotrophicum*, which was used in the experiments, differs from coenzyme F_{420} from *M. barkeri* in that it contains only two rather than 4 glutamyl residues [22].

The CHO-MFR dehydrogenase activity in *M. barkeri* was more sensitive towards cyanide inactivation than the enzyme system in *Mb. thermoautotrophicum* (fig.2).

4. DISCUSSION

Four aspects will be discussed here: (i) the presence of the CHO-MFR dehydrogenase activity in the pellet fraction; (ii) the physiological electron acceptor of the CHO-MFR dehydrogenase; (iii) the inactivation of CHO-MFR dehydrogenase by cyanide, and (iv) the function of the enzyme activity in methanol-grown *M. barkeri*.

4.1. *Association with the pellet fraction*

Cells of *Mb. thermoautotrophicum* and of *M. barkeri* are difficult to break. The cells have to be passed twice through a French pressure cell at 1100×10^5 Pa. Under these conditions, the cytoplasmic membrane is disrupted into very small pieces which sediment only at very high g values. Also, during this procedure, probably most of the peripheral membrane proteins are released into the soluble cell fraction. The finding that most of the CHO-MFR dehydrogenase activity was associated with the $160\,000\times g$ pellet is therefore of special interest. It indicates that the enzyme system either has a very high molecular mass or that it is tightly associated with the cytoplasmic membrane. The latter would support the proposal that in vivo the endergonic reduction of CO_2 to CHO-MFR with H_2 is driven by the electrochemical H^+ or Na^+ potential rather than by direct hydrolysis of ATP [15–17].

4.2. *The physiological electron acceptor*

Coenzyme F_{420} was the only physiological electron acceptor found to be used by the CHO-MFR dehydrogenase activity. The other coenzyme F_{420}-dependent enzyme known to be involved in CO_2-reduction to methane is methylene-H_4MPT dehydrogenase from *Mb. thermoautotrophicum* (strain ΔH) [23].

The $E^{\circ\prime}$ of the CO_2/CHO-MFR couple is -500 mV [13,15], that of the H^+/H_2 couple being -414 mV (see section 1). Thus, in vivo, CO_2 reduction with H_2 to CHO-MFR is probably energy-driven. The finding that the CHO-MFR dehydrogenase activity is associated with the pellet fraction indicates that the redox potential difference is overcome by the mechanism of reversed electron transport as has been shown for CO_2 reduction to CO ($E^{\circ\prime} = -520$ mV) in *M. barkeri* [25]. In this case, a membrane-associated component rather than coen-

zyme F_{420} would be the direct physiological electron acceptor and the reduction of coenzyme F_{420} with CHO-MFR could only be a side track.

4.3. *Inactivation by cyanide*

The finding that CHO-MFR dehydrogenase was inactivated by cyanide was surprising, since cyanide, at least in low concentrations, does not inhibit CO_2 reduction to CH_4 in cell suspensions of methanogenic bacteria [26,27]. Only the carbon monoxide dehydrogenase present in these bacteria was thought to be sensitive towards this poison. It therefore must be assumed that in intact cells the CHO-MFR dehydrogenase is protected by its substrates from cyanide inactivation.

Growth of methanogenic bacteria has been shown to be dependent on molybdenum [28]. The formate dehydrogenases of bacteria are known to be molybdo enzymes (e.g. [29,30]) which are inactivated by cyanide [31,32]. In analogy, it is tempting to speculate that CHO-MFR dehydrogenase could be a molybdenum-containing protein. Preliminary copurification studies support this hypothesis.

4.4. *Function in methanol-grown* M. barkeri

M. barkeri ferments CH_3OH according to the following equation:

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$$

One mol CH_3OH is oxidized to CO_2, the electrons being used for the reduction of 3 mol CH_3OH to CH_4. The pathway of CH_3OH oxidation to CO_2 has not yet been elucidated. Methanol-grown cells contain an F_{420}-dependent methylene-H_4MPT dehydrogenase, cyclohydrolase, and a CHO-MFR:H_4MPT formyltransferase (unpublished) [19]). The finding that the bacteria also contain a CHO-MFR dehydrogenase rather than a formate dehydrogenase indicates that methanol oxidation to CO_2 proceeds principally in reverse to the pathway used for CO_2 reduction to CH_4.

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