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Methyl-coenzyme M reductase preparations with high specific activity from H_2-preincubated cells of *Methanobacterium thermoautotrophicum*

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The study of the nickel enzyme methyl-coenzyme M reductase from methanogenic bacteria has been hampered until now by the fact that upon cell rupture the activity of the enzyme always dropped to at best only a few percent of its in vivo activity We describe here that when *Methanobacterium thermoautotrophicum* cells were preincubated with 100% H_2 before disintegration methyl-coenzyme M reductase activity stayed high The cell extracts with a specific activity of 2 U/mg protein exhibited two nickel-derived EPR signals, designated MCR-red1 and MCR-red2, previously only observed in intact cells The enzyme was purified 10-fold to a specific activity of 20 U/mg in the presence of methyl-coenzyme M, which stabilized both the activity and the EPR signal MCR-red1 The enzyme preparation displayed an UV/Vis spectrum with an absorption maximum at 386 nm and a shoulder at 420 nm Upon inactivation of the enzyme with O_2 or $CHCl_3$, the maximum at 386 nm and the EPR signals MCR-red1 and MCR-red2 disappeared

Nickel porphinoid, Coenzyme F_{430}, Methyl-coenzyme M reductase, EPR, *Methanobacterium thermoautotrophicum*

1. INTRODUCTION

Methyl-coenzyme M reductase catalyzes the reduction of methyl-coenzyme M (CH_3-S-CoM) with 7-mercaptoheptanoylthreonine phosphate (H-S-HTP) to CH_4 and the heterodisulfide of H-S-CoM and H-S-HTP This reaction is the methane-forming step in the energy metabolism of all methanogenic bacteria [1,2].

$$CH_3\text{-S-CoM} + \text{H-S-HTP} \rightarrow CH_4 + \text{CoM-S-S-HTP} \quad \Delta G^{\circ\prime} = -45 \text{ kJ/mol}$$

Methyl-coenzyme M reductase has a molecular mass of approximately 300 kDa. It is composed of three different subunits in an $\alpha_2\beta_2\gamma_2$ arrangement and contains two molecules of tightly, but not covalently, bound coenzyme F_{430} as chromophoric prosthetic group. Coenzyme F_{430} is a yellow nickel porphinoid of unique structure and properties [3,4]

Cell suspensions of *Methanobacterium thermoautotrophicum*, which grows on 80% H_2 and 20% CO_2, have been shown to exhibit three different EPR signals derived from methyl-coenzyme M reductase [5,6] Two of the signals, designated MCR-red1 and MCR-red2, were obtained by reduction of the cell suspension with 100% H_2. The third signal, MCR-ox1, was generated upon oxidation of the cells with 20% CO_2/80% N_2 Similar EPR signals have been reported for *Methanosarcina barkeri* [7]

Until now methyl-coenzyme M reductase has been purified only from cell extracts showing no EPR signal or the signal MCR-ox1 The extracts had no or only very little activity [8-11] which did not correlate with the intensity of the MCR-ox1 signal [6] The highest specific activity observed in extracts was 0 2 U/mg [11] which is only approximately 5% of the in vivo activity

We report here on the preparation of cell extracts showing the EPR signals MCR-red1 and MCR-red2 and having a specific methyl-coenzyme M reductase activity of 2 U/mg. The active enzyme was partially purified and characterized.

Abbreviations MCR, methyl-coenzyme M reductase, H-S-CoM or coenzyme M, 2-mercaptoethanesulfonate CH_3-S-CoM or methyl-coenzyme M, 2-(methylthio)ethanesulfonate, H-S-HTP, *N*-7-mercaptoheptanoylthreonine phosphate, CoM-S-S-HTP, mixed disulfide of H-S CoM and H-S-HTP, 1 U = 1 μmol/min, SDS-PAGE, sodium dodecylsulfate/polyacrylamide gel electrophoresis

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2. MATERIALS AND METHODS

M thermoautotrophicum (strain Marburg) (DSM 2133) was grown on 80% H_2/20% CO_2 with a doubling time of 1 5 h [10–12] The cells were harvested at the end of the exponential growth phase at a concentration of 1 5–2 g (dry mass) per liter Before harvest the cultures were vigorously gassed either with 100% H_2 (H_2-reduced cells) or with 20% CO_2/80% N_2 (CO_2-oxidized cells) for 20 min at 65°C and then cooled to 0°C

Cell extracts were prepared by sonification of 8 g freshly harvested

cells (wet mass) suspended in 16 ml 50 mM Tris-HCl, pH 7 6, at 0°C and 5% H_2/95% N_2 as gas phase Cell debris and membranes were removed by centrifugation at 160 000 × *g* at 8°C for 30 min Methyl-coenzyme M reductase was purified from extracts of H_2-reduced and CO_2-oxidized cells by fractionated ammonium-sulfate precipitation at 0°C under 5% H_2/95% N_2 as gas phase (see Table I) The enzyme preparations were kept at 0°C under 100% H_2

Methyl-coenzyme M reductase activity was assayed by following the formation of methane from CH_3-S-CoM at 60°C [10–12] The 0 4 ml assay mixture in 8 ml serum bottles contained 50 mM potassium phosphate, pH 7 0, 11 mM CH_3-S-CoM, 0 75 mM 7-mercaptoheptanoylthreonine phosphate, 15 mM dithiothreitol, 0 3 mM aquocobalamin, and 5-50 μg methyl-coenzyme M reductase The gas phase was H_2 at 2 × 10^5 Pa The reaction was started by increasing the temperature from 0°C to 60°C In time intervals of 3 min gas samples were withdrawn and analyzed for methane Protein was determined by the method of Bradford [13]

EPR spectra were recorded with a Varian E-3 EPR spectrometer operating at 9 6 GHz and 100 kHz field modulation

Quantification and simulation of EPR spectra were performed as in [6]

3 RESULTS

3 1. *Methyl-coenzyme M reductase from H_2-reduced cells*

Cell suspensions obtained from H_2-reduced cells showed the EPR signals MCR-red1 and MCR-red2 besides minor amounts of MCR-ox1 (Fig 1, A1) (for line shapes of the individual signals see [6]). Extracts from such cells showed the same signals (Fig 1, A2) and had an unusually high specific activity of methyl-coenzyme M reduction of 2 U/mg protein (Table I) Large MCR-red signals correlated with high specific activities and vice versa The two MCR-red signals and the activity were almost instantaneously abolished in the presence of trace amounts of O_2 On the contrary, the MCR-ox1 signal was not quenched by O_2 and was not correlated with the enzyme activity

When CH_3-S-CoM was added to the cell extract at 0°C the MCR-red2 signal disappeared and the MCR-red1 signal became larger (Fig 1, A3) Addition of H-S-CoM led to an increase in MCR-red2 leaving the MCR-red1 signal essentially unaltered (not shown)

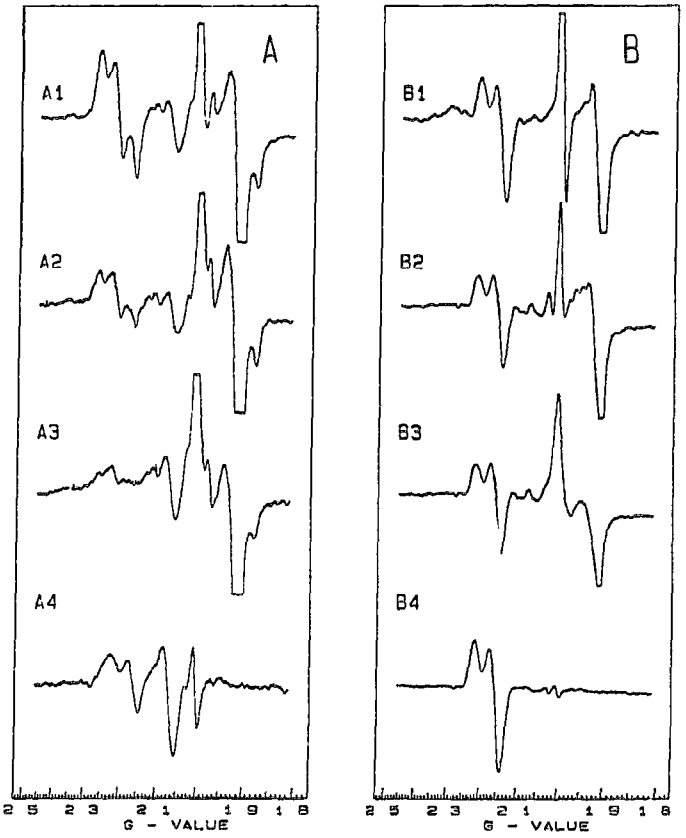

Fig 1 EPR signals from methyl-coenzyme M reductase of (A) H_2-reduced cells and (B) of CO_2-oxidized cells (1) Intact cells (35 mg protein/ml), (2) cell extract (20 mg/ml), (3) cell extract supplemented with 10 mM CH_3-S-CoM, (4) methyl-coenzyme M reductase preparation (5 mg/ml) in 50 mM Tris-HCl, pH 7 6 supplemented with 10 mM CH_3-S-CoM The EPR signal of the enzyme preparation from both the H_2-reduced and the CO_2-oxidized cells represented a spin concentration of 15–20% of the enzyme concentration EPR conditions microwave frequency, 9098 MHz, temperature, 77K, microwave power incident to the cavity 80 mW and modulation amplitude, 1 25 mT

Table I

Purification of methyl-coenzyme M reductase activity from *Methanobacterium thermoautotrophicum* by ammonium-sulfate precipitation

Before addition of ammonium-sulfate the cell extract was supplemented with 10 mM CH_3-S-CoM The 100% ammonium-sulfate pellet, which is referred to as methyl-coenzyme M reductase preparation, was dissolved to a final concentration of 5 mg protein/ml in 50 mM Tris-HCl (pH 7 6) supplemented with 10 mM CH_3-S-CoM

	Purification from H_2-reduced cells			CO_2-oxidized cells		
	Activity (U)	Protein (mg)	Specific activity (U/mg)	Activity (U)	Protein (mg)	Specific activity (U/mg)
Cell extract	981 9	473 0	2 1	84 0	437 5	0 19
70% ammonium-sulfate supernatant	1137 5	94 3	12 1	56 7	108 5	0 52
70% ammonium-sulfate pellet	246 6	425 9	0 58	16 1	254 8	0 06
100% ammonium-sulfate supernatant	416 0	45 5	9 1	42 0	69 3	0 61
100% ammonium-sulfate pellet	556 1	27 7	20 1	27 3	37 8	0 72

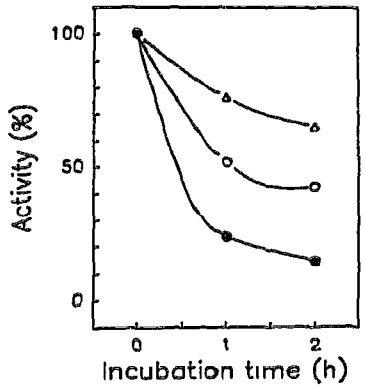

Fig 2 Protection of methyl-coenzyme M reductase from inactivation at 60°C in the presence of CH_3-S-CoM Cell extract (1 ml samples) was supplemented with 5 mM CH_3-S-CoM or 5 mM H-S-CoM as indicated The samples were incubated in 8 ml serum bottles under 2 × 10^5 Pa H_2 at 60°C At the times indicated 10 μl samples were withdrawn and tested for activity (● Cell extract, (△) cell extract supplemented with 5 mM CH_3-S-CoM, (○) cell extract supplemented with 5 mM H-S-CoM

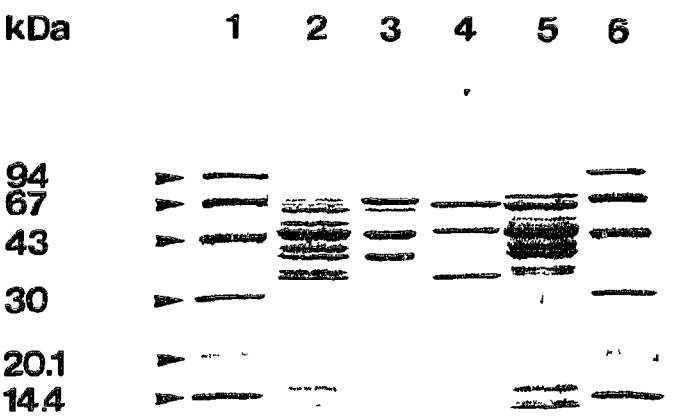

Fig 3 SDS-PAGE of the methyl-coenzyme M reductase preparations purified as described in Table I Proteins in 0 1% SDS were separated on a 12% polyacrylamide slab gel (8 cm × 7 cm) which was subsequently stained with Coomassie brilliant blue R250 (Lanes 1 and 6) molecular mass standard (Pharmacia, Freiburg, Germany, (lane 2) 60 μg protein of the enzyme preparation from CO_2-oxidized cells, (lane 5) 80 μg protein of the enzyme preparation from H_2-reduced cells (lane 3) 30 μg protein of pure methyl-coenzyme M reductase I, (lane 4) 30 μg protein of pure methyl-coenzyme M reductase II

Both CH_3-S-CoM and H-S-CoM were found to stabilize the high enzyme activity, CH_3-S-CoM being somewhat more effective than H-S-CoM (Fig 2). Therefore, in the following purification procedure all buffers were supplemented with CH_3-S-CoM (10 mM)

Purification of the highly active enzyme was achieved by ammonium-sulfate precipitation, exploiting the unusual property of methyl-coenzyme M reductase to be soluble even at very high ammonium-sulfate concentrations In the experiment described in Table I a 10-fold enriched enzyme preparation was obtained with a specific activity of 20 U/mg SDS-PAGE revealed that the preparation was a mixture of the two isoenzymes MCR I (Fig. 3, lane 3) and MCR II (Fig 3, lane 4) [12] and still contained some contaminating proteins (Fig 3, lane 5) The impurities contributed to less than 10% of the total protein as estimated by photometric scans of the Coomassie brilliant blue stained gels (not shown) The N-terminal amino acid sequence of the 16 kDa impurity showed no sequence homology to one of the two open reading frames of unknown function in the methyl-coenzyme M reductase operon [14] The methyl-coenzyme M reductase preparation contained also some coenzyme F_{420} Attempts to further purify the reductase resulted in a 90% loss in specific activity. Purification steps tried were chromatography on phenyl-superose, Mono-Q, and Sephadex G25 at 20°C

The highly active enzyme preparation showed the MCR-red1 signal besides minor amounts of MCR-ox1 signal (Fig. 1 A4) The UV/Vis spectrum exhibited an absorption maximum at 386 nm and a shoulder at 420 nm (Fig 4A) The EPR signal MCR-red1 and the absorption maximum at 386 nm disappeared and the activity drastically decreased upon contact of the enzyme with either air or $CHCl_3$ The parallel decrease in MCR-

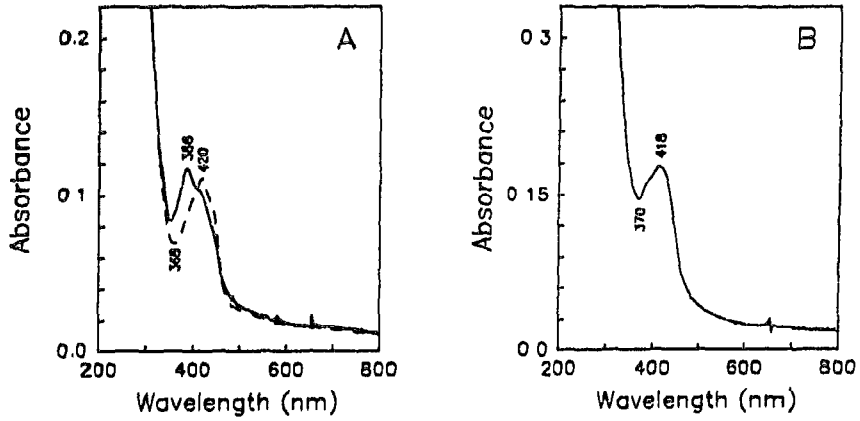

Fig 4 UV/vis spectrum of methyl-coenzyme M reductase purified as described in Table I from H_2-reduced cells (A) and from CO_2-oxidized cells (B) The dashed line in Fig 4A shows the spectrum after addition of $CHCl_3$ (1 7 μmol to the 1 ml enzyme solution) The protein concentration in (A) was 0 6 mg/ml and in (B) 1 0 mg/ml

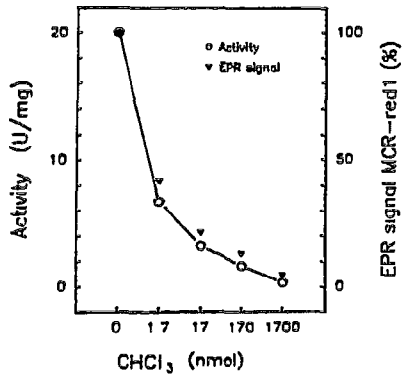

Fig 5 Inactivation by $CHCl_3$ of methyl-coenzyme M reductase partially purified from H_2-reduced cells and the parallel decrease of the EPR signal MCR-red1 To 0 6 ml enzyme solution (5 mg/ml) in an 8 ml serum vial at 0°C gaseous $CHCl_3$ was added in the amounts indicated After 1 min equilibration of the gaseous and the liquid phase by gentle shaking a 5 μl sample was withdrawn for the assay of enzyme activity and a 400 μl sample was transferred into an EPR tube for the registration of the EPR signal The amplitude of the $g_{x,y}$-line at g_x = 2 06 was measured

red1 signal and activity in the presence of $CHCl_3$ is shown in Fig 5

3 2. *Methyl-coenzyme M reductase from CO_2-oxidized cells*

Cell suspensions obtained after preincubation of the growing culture with 20% CO_2/80% N_2 showed only the MCR-ox1 signal (Fig. 1, B1) Extracts from such cells showed the same signal (Fig. 1, B2) and had only very little methyl-CoM reductase activity (Table I). The EPR signal and the activity did not correlate and were not affected by the addition of CH_3-S-CoM (Fig 1, B3) and H-S-CoM (not shown) Via the purification procedure described in Table I only a 4-fold purification to a specific activity of 0.72 U/mg was achieved The enzyme preparation displayed the MCR-ox1 signal (Fig 1, B4), which was not affected by either O_2 or $CHCl_3$ The SDS-PAGE pattern (Fig. 3, lane 2) was indistinguishable from the pattern obtained for the H_2-reduced cells (Fig 3, lane 5). The enzyme preparation differed, however, from the preparation from H_2-reduced cells in that it did not show the absorption maximum at 386 nm (Fig 4B).

The enzyme preparation from CO_2-oxidized cells, as the enzyme preparation from H_2-reduced cells, was a mixture of the two isoenzymes MCR I and MCR II (Fig 3, lanes 2 and 5) After separation by chromatography on Mono Q HR 10/10 as described in [12] both isoenzymes displayed identical MCR-ox1 signals (data not shown).

4 DISCUSSION

The catalytic mechanism of methyl-coenzyme M reductase has been proposed to involve coenzyme F_{430} in the Ni(I) form [15,16] The finding that the enzyme is only highly active in the MCR-red1 or MCR-red2 form supports this mechanism since these two signals are very probably derived from Ni(I) This is evidenced by the following observations. (i) The two MCR-red signals were quenched by O_2 and $CHCl_3$ with concomitant loss in activity Both potent inhibitors of methanogenesis rapidly oxidize coenzyme F_{430} in the Ni(I) form [17,18] (ii) The EPR signal MCR-red1 is similar to the EPR signal displayed by coenzyme F_{430} in the Ni(I) form rather than to the signal of the Ni(III) form [16,17]. (iii) The highly active methyl-coenzyme M reductase showed an absorption peak at 386 nm which is closer to the absorption peak at 382 nm of Ni(I)F_{430} than to the absorption peak at 368 nm of Ni(III)F_{430} [16,17]. (iv) Completely inactive methyl-coenzyme M reductase from *M thermoautotrophicum* (strain ΔH), has been reported to be photoreactivated to a specific activity of 1 mU/mg which was interpreted to indicate that Ni(I) is involved in the catalytic mechanism since the excited state is predicted to lead to the formation of Ni(I) [9]

The existence of two signals derived from Ni(I) is explainable assuming a change at the axial position in the coordination of nickel in F_{430} bound to the enzyme The finding that CH_3-S-CoM increased the MCR-red1 signal and H-S-CoM the MCR-red2 signal is in agreement with this interpretation

If the signals MCR-red1 and MCR-red2 are derived from Ni(I) what might be the source of the signal MCR-ox1? It could be either Ni(III) or Ni(I) in a stable form It has e.g been reported that Ni(I) can be stabilized in the presence of air if complexed by interlocked macrocyclic ligands [19]

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