Single step purification of methylenetetrahydromethanopterin reductase from *Methanobacterium thermoautotrophicum* by specific binding to Blue Sepharose CL-6B

K. Ma and R.K. Thauer

Laboratorium für Mikrobiologie, Fachbereich Biologie, Philipps-Universität, Karl-von-Frisch-Straße, D-3550 Marburg/Lahn, FRG

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Methylenetetrahydromethanopterin reductase from metanogenic archaebacteria catalyzes the reversible reduction of N^5,N^{10}-methylenetetrahydromethanopterin to N^5-methyltetrahydromethanopterin with reduced coenzyme F_{420} as electron donor. The enzyme is involved in methane formation from CO_2 and in methanol disproportionation to CO_2 and CH_4. We report here that the reductase from *Methanobacterium thermoautotrophicum* specifically binds to Blue Sepharose CL-6B. Binding was competitive with coenzyme F_{420} rather than with NAD, NADP, FAD, FMN, AMP, ADP and ATP. The reductase could also be desorbed with salt. Based on this property an affinity chromatographic procedure for the purification of the enzyme was developed.

Methanobacterium thermoautotrophicum; Methanogenesis; Tetrahydromethanopterin; Coenzyme F_{420}; Affinity chromatography; Blue Sepharose CL-6B

1. INTRODUCTION

The reversible reduction of N^5, N^{10}-methylenetetrahydromethanopterin ($CH_2=H_4MPT$) to N^5-methyltetrahydromethanopterin (CH_3–H_4MPT) with reduced coenzyme F_{420} ($F_{420}H_2$) is a central step in the energy metabolism of methanogenic archaebacteria [1].

$$CH_2=H_4MPT + F_{420}H_2 \rightleftharpoons CH_3\text{-}H_4MPT + F_{420}$$
$$\Delta G^{\circ\prime} = -5.2 \text{ kJ/mol}$$

The reaction is catalysed by methylenetetrahydromethanopterin reductase ($CH_2=H_4MPT$ reductase). The enzyme has been purified from *Methanobacterium thermoautotrophicum* strains ΔH [2] and Marburg [3] and from *Methanosarcina barkeri* [4]. It is composed of 4 identical subunits with an apparent molecular mass of 36 kDa. A prosthetic group is lacking. The enzyme is highly specific for coenzyme F_{420}. Neither NADH or NADPH nor $FADH_2$ or $FMNH_2$ can substitute for the reduced 5′-deazaflavin. (For structures of F_{420} and $F_{420}H_2$, see Fig. 1).

During gel filtration experiments we observed that $CH_2=H_4MPT$ reductase was tightly bound by the Dextrane Blue (M_r = 2 000 000), which was used to calibrate the columns. An analysis of this phenomenon revealed that the Dextrane Blue specifically binds to the coenzyme F_{420} binding site of the reductase.

Correspondence address: R.K. Thauer, Laboratorium für Mikrobiologie, Fachbereich Biologie, Philipps-Universität, Karl-von-Frisch-Straße, D-3550 Marburg/Lahn, FRG

2. MATERIALS AND METHODS

Dextrane Blue (M_r ca. 2 000 000) stained by covalent binding with Cibacron Blue F3G-A was from Serva (Heidelberg, FRG). Blue Sepharose CL-6B, which is Cibacron Blue F3G-A attached to the cross-linked agarose gel Sepharose CL-6B, was from Pharmacia (Freiburg, FRG). The concentration of coupled dye is approximately 7 μmol/g freeze-dried powder. Coenzyme F_{420} [5] and tetrahydromethanopterin [6] were isolated from *Methanobacterium thermoautotrophicum* (strain Marburg, DSM 2133), which was obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, FRG). *M. thermoautotrophicum* was grown at 65°C on 80% H_2/20% CO_2 [7]. After harvest the cells were stored at -80°C.

2.1. *Enzyme purification*

Cell extract of *M. thermoautotrophicum* was prepared by suspending 5 g cells (wet mass) in 20 ml 50 mM Tris-HCl, pH 7.7, containing 0.2 mg DNase I. The suspension was passed 3–4 times through a French pressure cell at 110 MPa and then centrifuged at 37 000 × *g* for 30 min at 7°C. The supernatant contained 100% of the methylenetetrahydromethanopterin reductase activity.

After centrifugation of the cell extract for 60 min at 100 000 × *g* the supernatant was diluted 5-fold with 50 mM Tris-HCl, pH 7.7. A 25 ml sample was then applied to a Blue Sepharose CL-6B column (1.5 × 6 cm) equilibrated with 50 mM Tris-HCl, pH 7.7. The flow rate was 1 ml/min. The column was washed with buffer containing KCl: 30 ml, 0.2 M; 40 ml, 0.27 M; 40 ml, 0.6 M; 40 ml, 0.9 M; and 100 ml, 2 M. The reductase activity was recovered in the fractions eluted with 0.6 M KCl. The fractions containing reductase activity were pooled (20 ml), concentrated to 2 ml, and desalted by ultrafiltration (PM 30 filter, Amicon, Witten, FRG). The desalted solution was reapplied to a Blue Sepharose CL-6B column (1.5 cm × 6 cm), which was equilibrated and eluted as described above. After concentration and desalting by ultrafiltration the enzyme solution (1 mg protein per ml of 50 mM Tris-HCl, pH 7.7) was stored at −20°C. Under these conditions practically no activity was lost within two months.

All purificication steps were performed at room temperature. The Blue Sepharose column was run in an anaerobic chamber (Coy, Ann

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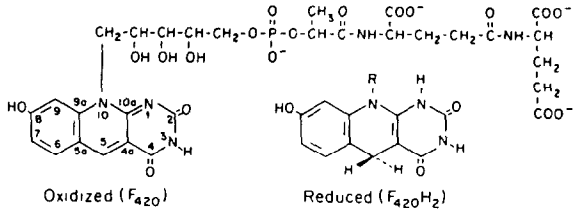

Fig. 1. Structure of coenzyme F_{420} in the oxidized (F_{420} and in the reduced form ($F_{420}H_2$) [1].

Arbor, MI, USA). It turned out, however, that the reductase was also stable under aerobic conditions.

Protein was determined by using the Bradford microassay [8,9].

2.2. *Enzyme activity*

The enzyme activity was determined at 55°C in 1.5 ml glass cuvettes with N_2 as gas phase [3]. The 1 ml assay mixture routinely contained: 100 mM Tris-HCl, pH 7.7 (adjusted to this pH at room temperature), 10 mM mercaptoethanol and 16 μM coenzyme F_{420}. $Na_2S_2O_4$ (3 μmol) was added to reduce F_{420}, which at pH 7.7 and 55°C took 3–4 min. Then 15 μmol formaldehyde was added which quenched the excess dithionite. After another minute, 16 nmol H_4MPT was injected. Formaldehyde and H_4MPT spontaneously reacted to $CH_2=H_4$MPT under the experimental conditions [10]. The $CH_2=H_4$MPT reductase reaction was started with protein. $F_{420}H_2$ oxidation to F_{420} was followed at 420 nm (ϵ_{420} = 38 $mM^{-1} \cdot cm^{-1}$). For the determination of enzyme activity in the cell extract the assays were performed with 40% N_2/60% acetylene in the gas phase. Acetylene was used to inhibit $F_{420}H_2$ oxidation via the F_{420}-reducing hydrogenase, which was present in the cell extracts [11].

2.3. *Desorption experiments*

The assays were performed at room temperature and under aerobic conditions in 2 ml plastic tubes containing a small magnetic paddle. Enzyme solution (0.7 ml; 50 μg purified protein) was mixed with Blue Sepharose gel (0.3 ml; ≈ 40–120 mg freeze-dried powder) and incubated for 15 min. The pH was 7.7 (50 mM Tris-HCl). After centrifugation at 11 000 × *g* for 1 min the enzyme activity was determined in a sample of the supernatant. Then either coenzyme F_{420} or KCl were added. After resuspension, incubation for 15 min, and recentrifugation the activity was determined again. The percentage of enzyme desorbed was calculated from the activity in the supernatant and the activity added to the assay.

3. RESULTS AND DISCUSSION

Methylenetetrahydromethanopterin reductase from *M. thermoautotrophicum* migrated on gel filtration columns with an apparent molecular mass of between 130 kDa and 160 kDa [3]. When the enzyme was applied to the columns together with Dextrane Blue the apparent molecular mass increased to 2000 kDa. This finding indicated that the reductase was tightly bound to Dextrane Blue (M_r = 2 000 000) under the experimental conditions.

3.1. *Specific binding of the reductase to Blue Sepharose*

To determine whether binding was specific or not we studied the adsorption of the reductase to Blue Sepharose CL-6B. The Sepharose gel contains covalently bound the same chromophore, Cibacron Blue, as Dextrane Blue but has a much higher molecular mass

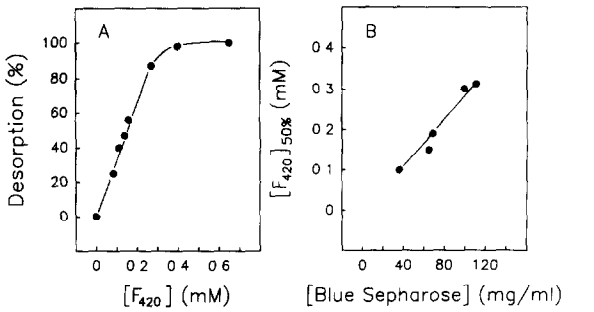

Fig. 2. Desorption of methylenetetrahydromethanopterin reductase from Blue Sepharose CL-6B with coenzyme F_{420}. (A) Dependence of desorption on the coenzyme F_{420} concentration. The Blue Sepharose concentration was 75 mg/ml. (B) Dependence of the F_{420} concentration required for 50% desorption $[F_{420}]_{50\%}$ on the Blue Sepharose concentration. For assay conditions see section 2.

and can therefore easily be separated from solutes by centrifugation. The purified reductase in 50 mM Tris-HCl, pH 7.7, was mixed with Blue Sepharose at room temperature for 15 min and the gel was then centrifuged off. The supernatant contained no reductase activity indicating that all of the enzyme was adsorbed to the Blue Sepharose.

Blue Sepharose is known to bind reversibly many proteins, most but not all of which require adenylyl-containing coenzymes such as NAD or ATP [12]. Some of these can be desorbed by low concentrations of the free cofactor. Studies using X-ray crystallography show that Cibacron Blue binds to the nucleotide-binding pocket [12]. We therefore determined whether coenzyme F_{420} or one of the adenylyl-containing substances NAD, NADP, FAD, AMP, ADP and ATP were able to desorb the reductase from Blue Sepharose. Concentrations up to 5 mM were tested. Coenzyme F_{420} rather than the other compounds was found to be effective. Apparently the Cibacron Blue chromophore specifically binds to the coenzyme F_{420} binding site of methylenetetrahydromethanopterin reductase from *M. thermoautotyrophicum*.

3.2. *Competition of binding with coenzyme F_{420}*

The coenzyme F_{420} concentration, $[F_{420}]_{50\%}$, was

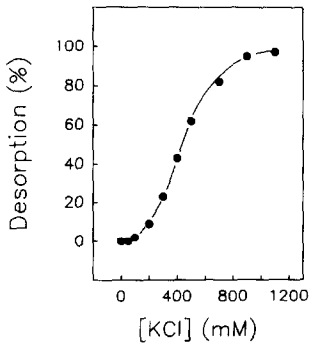

Fig. 3. Desorption of methylenetetrahydromethanopterin reductase from Blue Sepharose CL-6B with salt. For assay conditions see section 2. The Blue Sepharose concentration was 75 mg/ml.

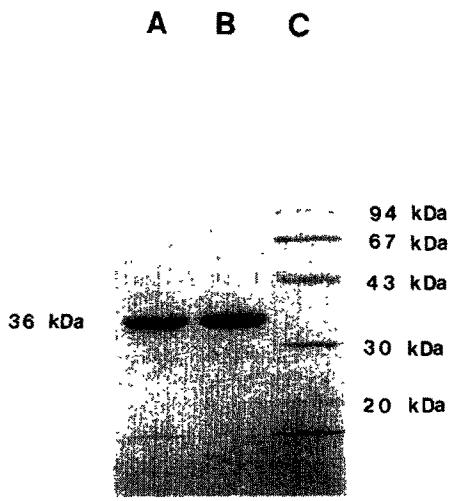

Fig. 4. Analysis of purified methylenetetrahydromethanopterin reductase from *M. thermoautotrophicum* by sodium dodecylsulfate polyacrylamide gel electrophoresis. Protein in sodium dodecylsulfate (0.1%) was separated on 12.5% polyacrylamide slab gels (8 cm × 7 cm), which were subsequently stained with Coomassie brilliant blue R 250. (Lane A) 1 μg reductase after the first chromatography on Blue Sepharose CL-6B (Lane B) 1 μg reductase after rechromatography on Blue Sepharose CL-6B. (Lane C) Protein-standard mixture (5 μg protein): phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; ovalalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa.

determined at which 50% of the enzyme activity was desorbed from Blue Sepharose. $[F_{420}]_{50\%}$ was found to be 0.2 mM at a Blue Sepharose concentration of 75 mg/ml ≃ 0.5 μmol coupled dye/ml) (Fig. 2A). $[F_{420}]_{50\%}$ was 0.1 mM when half the amount of Blue Sepharose was used in the assay and 0.4 mM when twice the amount was employed (Fig. 2B). $[F_{420}]_{50\%}$ thus linearly increased with the Blue Sepharose concentration, [Blue Sepharose]. Such a dependence is to be expected when the binding of Blue Sepharose and of coenzyme F_{420} to the reductase is mutually exclusive.

$$\text{Blue Sepharose} + F_{420}\cdot\text{Reductase} \rightleftharpoons F_{420} + \text{Blue Sepharose}\cdot\text{Reductase}$$

$$K = \frac{[F_{420}] \times [\text{Blue Sepharose}\cdot\text{Reductase}]}{[\text{Blue Sepharose}] \times [F_{420}\cdot\text{Reductase}]}$$

When $[F_{420}] = [F_{420}]_{50\%}$ then [Blue Sepharose · Reductase] = [F_{420} · Reductase] and $[F_{420}]_{50\%} = K \cdot$ [Blue Sepharose].

3.3. *Desorption with salt*

Desorption of the reductase from Blue Sepharose could also be achieved with salt albeit only at high concentrations. A concentration dependence is given in Fig. 3 showing that 0.6 M KCl was required for 50% dissociation of the Blue Sepharose reductase complex. Other proteins present in cell extracts of *M. thermoautotrophicum*, which were non-specifically adsorbed to Blue Sepharose, were found to be eluted at much lower KCl concentrations. The following purification procedure is based on these findings.

3.4. *Purification procedure*

Cell extract of *M. thermoautotrophicum* was applied to a Blue Sepharose column, which was subsequently washed with a KCl step gradient. The non-specifically bound proteins eluted first and then methylenetetrahydromethanopterin reductase. In this single step a 50-fold purification with a yield of 87% was achieved (Table I). At this stage the enzyme was already almost 70% pure (Fig. 4, lane A). Upon rechromatography on Blue Sepharose an apparently homogenous preparation was obtained as revealed by sodium dodecylsulfate polyacrylamide gel electrophoresis (Fig. 4, lane B). The overall yield was 67%. Previous purification procedures involved fast protein liquid chromatography of the enzyme on DEAE-Sepharose (fast flow), Mono-Q HR, Phenyl-Superose HR, and Superose [3].

3.5. *Enzyme from* Methanosarcina barkeri

We investigated whether the methylenetetrahydromethanopterin reductase from *Methanosarcina barkeri.* [4] could be purified by the same procedure. It was found that also this enzyme tightly binds to Blue Sepharose. However, the binding was so tight that elution was possible only at KCl concentrations above 1 M. The recovery was less than 50% indicating that binding was associated with inactivation of the enzyme.

Table I

Purification by affinity chromatography of methylenetetrahydromethanopterin reductase from *Methanobacterium thermoautotrophicum*

Purification step	Protein (mg)	Activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell extract	114	228	2	1	100
Blue Sepharose	2.1	197	94	47	87
Blue Sepharose (rechromatography)	1.1	154	140	70	67

Cell extract (5 ml) was prepared from 1 g cells (wet mass) and methylenetetrahydromethanopterin reductase was purified by specific binding to Blue Sepharose CL-6B as described in section 2. 1 U = 1 μmol/min.

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