

Purification of the Coenzyme F₄₂₀-reducing Hydrogenase from *Methanobacterium formicicum*

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All carbon-dioxide-reducing methanogenic microbes utilize H₂ as an electron donor ($4\text{H}_2 + \text{CO}_2 = \text{CH}_4 + 2\text{H}_2\text{O}$). Many have two distinct hydrogenases (Jin et al. 1983; Nelson et al. 1984; Jacobson et al. 1992): (1) The F₄₂₀ hydrogenase reduces coenzyme F₄₂₀ (F₄₂₀) and methyl viologen and (2) the methyl viologen hydrogenase (also referred to as the F₄₂₀-nonreactive hydrogenase) reduces methyl viologen but not F₄₂₀. F₄₂₀ hydrogenases from several H₂-oxidizing methanogens have been purified and characterized (Jacobson et al. 1982; Yamazaki 1982; Fauque et al. 1984; Fox et al. 1987; Muth et al. 1987; Sprott et al. 1987; Baron and Ferry 1989; Fiebig and Friedrich 1989). Each forms large aggregates with molecular weights of 720,000–1,300,000 and each contains nickel and iron-sulfur centers. Flavin adenine dinucleotide (FAD), which is required for reduction of F₄₂₀ (Fox et al. 1987; Nelson et al. 1987; Baron and Ferry 1989), is present in the enzymes from *Methanobacterium thermoautotrophicum*, *Methanobacterium formicicum*, and *Methanococcus voltae*. Most F₄₂₀ hydrogenases are associated with the cytoplasmic membrane. The best-studied is the F₄₂₀ hydrogenase from *M. thermoautotrophicum*; however, the enzyme from *M. formicicum* is nearly identical, and the purification protocol presented here is generally applicable to the purification of most F₄₂₀ hydrogenases.

The *M. formicicum* strain JF-1 (DSM 2639) used here was isolated from a benzoate-degrading consortium originally derived from sewage sludge.

MATERIALS

Cells from harvested cultures (cell paste) of *Methanobacterium formicicum* grown with H₂ plus CO₂ (see Protocol 18).

Serum-stoppered cuvette

French pressure cell (SLM Aminco)

DEAE-cellulose column (5 x 12 cm; Whatman DE-53)

Phenyl-Sepharose CL-4B column (2.6 x 10 cm; Pharmacia 17-0810-02)

Mono Q HR 10/10 anion-exchange column (Pharmacia 17-0556-01)

Electrophoretic concentrator (ISCO model 1750)

FPLC (fast protein liquid chromatography) system (Pharmacia 18-1040-00)

Electrophoresis apparatus with 4% polyacrylamide slab gels

Triton X-100 (Sigma X-100)

Buffer A	
potassium TES (<i>N</i> -tris[hydroxymethyl]-methyl-2-aminoethane sulfonic acid) (pH 7.5)	75 mM
MgCl ₂	15 mM
2-mercaptoethanol (Sigma M 6250)	3 mM
DNase I (Sigma D 4263)	10 µg/ml
Buffer B	
potassium TES (pH 7.5; Fisher BP309)	50 mM
MgCl ₂	10 mM
2-mercaptoethanol	2 mM
5% (v/v) glycerol	
Buffer C	
asparagine-Tris (pH 7.3)	34 mM
FAD (flavin adenine dinucleotide)	2.5 µM
2.5% (v/v) glycerol	
Buffer D	
Tris-chloride (pH 7.5)	1 mM
Assay reaction mixture (0.5 ml)	
potassium phosphate (pH 7.5)	50 mM
2-mercaptoethanol	20 mM
F ₄₂₀	48 µM
<i>or</i>	
methyl viologen	20 mM

Caution: 2-Mercaptoethanol may be fatal if inhaled or absorbed through the skin and is harmful if swallowed. High concentrations are extremely destructive to the mucous membranes, upper respiratory tract, skin, and eyes. Wear gloves and safety glasses and work in a chemical fume hood.

Methyl viologen may be fatal if inhaled, swallowed, or absorbed through skin. It is irritating to mucous membranes and upper respiratory tract. Wear gloves and safety glasses and work in a chemical fume hood.

METHODS

The protocols that follow are adapted from Baron and Ferry (1989).

Assay

The purification is performed without precautions to exclude oxygen and yields inactive protein at each step.

1. Reactivate the enzyme by adding (final concentration) 48 µM F₄₂₀ and 30 µM FAD, vacuum degassing the solution with N₂, flushing the headspace with H₂ for 1 minute, and incubating for 30–60 minutes at 35°C. Reactivation in the absence of FAD results in loss of F₄₂₀-dependent activity relative to methyl-viologen-dependent activity.
2. Make the reaction mixture anaerobic by vacuum degassing with N₂ and then transfer the mixture to a serum-stoppered cuvette containing an atmosphere of 82 KPa H₂. Assay H₂ uptake activity spectrophotometrically at 35°C by following the reduction of F₄₂₀ ($\epsilon_{420} = 42.5 \text{ mM}^{-1} \text{ cm}^{-1}$ at pH 7.5) or methyl viologen ($\epsilon_{603} = 11.3 \text{ mM}^{-1} \text{ cm}^{-1}$). A unit of activity is the reduction of 1 µmole of acceptor min⁻¹.

Purification

Prepare cell extract anaerobically as described in Protocol 18. Perform all subsequent steps without anaerobic precautions and at 4°C.

1. Thaw the cell paste under a stream of N₂ and resuspend in twice its weight of vacuum-degassed Buffer A. Pass the resuspended cells once through the French pressure cell at 138 MPa (20,000 lb/in²). Adjust the lysate to 2% (v/v) in Triton X-100, mix gently for 1 hour on a rocking platform, and centrifuge at 30,000g for 20 minutes at 5°C.
2. Load the extract onto a DEAE-cellulose column previously equilibrated with Buffer B. Wash the column with 1.5 bed volumes of Buffer B and develop with a linear gradient of KCl (0–0.5 M, 5 bed volumes). Elute F₄₂₀-hydrogenase in 0.18–0.28 M KCl and methyl viologen hydrogenase in 0.28–0.41 M KCl.
3. Pool fractions from step 2 that have a high ratio of F₄₂₀-dependent to methyl-viologen-dependent activity and adjust to 40% saturation (0°C) with (NH₄)₂SO₄. Centrifuge at 19,000g for 30 minutes. Adjust the supernatant solution to 70% saturation (final concentration) with (NH₄)₂SO₄ and centrifuge at 19,000g for 30 minutes.
4. Resuspend the protein pellet from step 3 in 60 ml of Buffer B containing 1 M KCl and load onto phenyl-Sepharose CL-4B column previously equilibrated with the same buffer. Wash the column (2 ml/minute) with 4 bed volumes of buffer and elute hydrogenase activity with 3 bed volumes of a linear gradient decreasing from 1 M to 0 M KCl and simultaneously increasing from 0% to 1.5% (v/v) Triton X-100.
5. Pool active fractions from step 4 and apply to a Mono Q column previously equilibrated with Buffer A. Wash the column (2 ml/minute) with 30 ml of Buffer A containing 0.25 M KCl and elute hydrogenase with a linear gradient of KCl (0.25–0.60 M, 140 ml).
6. Pool and dialyze active fractions from step 5 overnight against 1 liter of Buffer C. Apply samples (3 mg of protein) to 3-mm thick, 4% polyacrylamide slab gels and electrophorese at 30 mA constant current. Excise the region containing the brown hydrogenase band ($R_f = 0.40$) from the gels, mince into small pieces, and elute into Buffer D using the electrophoretic concentrator. Adjust the purified enzyme to 5% (v/v) in glycerol and freeze in liquid N₂. The purified enzyme can also be stored in air at –20°C for at least 1 month without loss of activity.

COMMENTARY

- Native gradient gel electrophoresis of the purified enzyme reveals two protein bands that stain for F₄₂₀-dependent activity (Baron and Ferry 1989). The high-molecular-weight band contains approximately fourfold more protein than the lower-molecular-weight band; however, two-dimensional native SDS-polyacrylamide gel electrophoresis indicates the same $\alpha_1\beta_1\tau_1$ subunit composition (with molecular weights of 43,600, 36,700, and 28,800) for both species. The results suggest that the F₄₂₀-hydrogenase is purified to

electrophoretic homogeneity primarily as an aggregate of a 109,000-molecular-weight F_{420} -reducing species. These results are similar to those reported for the F_{420} -reducing hydrogenase purified from *M. thermoautotrophicum* (Fox et al. 1987).

- For methods that describe the purification of methyl viologen hydrogenases from methanogens, see Jin et al. (1983) and Reeve et al. (1989).

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