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Isolation and characterization of polyferredoxin from *Methanobacterium thermoautotrophicum*

The *mvhB* gene product of the methylviologen-reducing hydrogenase operon

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The methylviologen-reducing hydrogenase operon of *Methanobacterium thermoautotrophicum* contains an open reading frame, *mvhB*, the product of which was predicted to have a molecular weight of 44 kDa and to contain as many as 48 iron atoms in 12 [4Fe–4S] clusters, and was therefore suggested to be a polyferredoxin. We have now, for the first time, isolated this polyferredoxin. Its identity with the *mvhB* gene product was evidenced by a comparison of the N-terminal amino acid sequence. The dark-brown protein of apparent molecular weight 44 kDa was found to contain 53 mol Fe and 43 mol acid-labile sulfur per mol. The UV/visible spectrum showed two maxima at 280 nm and 390 nm, and a shoulder at 308 nm. The A_{390}/A_{280} ratio was 0.73. The molar extinction coefficient at 390 nm was 170,000 $M^{-1} \cdot cm^{-1}$. In the dithionite reduced state the protein displayed an EPR spectrum like that of [4Fe–4S] clusters. The results indicate that the *mvhB* gene product is indeed a polyferredoxin.

Polyferredoxin; Iron-sulfur protein; Hydrogenase; Methanogenic bacteria; *Methanobacterium thermoautotrophicum*

1. INTRODUCTION

Methanobacterium thermoautotrophicum is a thermophilic archaebacterium that grows on H_2 and CO_2 while producing methane [1,2]. The organism contains two Ni–Fe hydrogenases [3,4], a coenzyme F_{420}-reducing hydrogenase [5] and a methylviologen-reducing hydrogenase (MV-hydrogenase), the physiological electron acceptor of which is unknown [6].

The MV-hydrogenase of *M. thermoautotrophicum*, as isolated, is composed of three subunits of apparent molecular weights 52, 41 and 17 kDa, respectively [6,7]. The three genes *mvhDGA* encoding these three polypeptides have been cloned and sequenced. They have been found to be organized in an operon which contains a fourth open reading frame designated *mvhB*. From the deduced amino acid sequence, the *mvhB* gene product was predicted to have a molecular weight of 44 kDa and to contain six tandemly repeated clostridial ferredoxin-like domains and was therefore predicted to be a polyferredoxin that could contain as many as 48 iron atoms in 12 [4Fe–4S] clusters [8].

The *mvhB* gene has also been found in the MV-hydrogenase operon of *Methanothermus fervidus* [9] and *Methanococcus voltae* [10] and shown to be conserved. It was therefore postulated that the function of *mvhB* gene product must somehow be associated with that of the MV-hydrogenase. Until now this gene product has, however, not been detected in any of the methanogenic bacteria investigated. Its isolation from *M. thermoautotrophicum* (strain Marburg) and characterization are described here.

Abbreviations: MV-hydrogenase, methylviologen-reducing hydrogenase; SDS-PAGE, sodiumdodecyl sulfate polyacrylamide gel electrophoresis; DTT, dithiothreitol; CAPSO, (3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid)

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2. MATERIALS AND METHODS

All column materials and FPLC columns were from Pharmacia (Freiburg, Germany). YM 30 membranes, Centricon 30 and Centricon 100 microconcentrators were from Amicon (Witten, Germany).

M. thermoautotrophicum (strain Marburg DSM 2133) was grown, harvested, and cell extracts were prepared from 20 g cells (2 g protein) as described [7]. The cell extract (50 ml, 1.8 g protein) was centrifuged anaerobically at 160,000 × *g* for 30 min at 8°C. All subsequent purification steps were performed in an anaerobic chamber at 18°C [7]. All buffers used contained 2 mM dithiothreitol.

The 160,000 × *g* supernatant (45 ml, 1.2 g protein) was diluted 1:2 with 50 mM Tris/HCl, pH 7.6, and applied to a DEAE Sepharose fast flow column (2.6 × 15 cm) equilibrated with 50 mM Tris/HCl, pH 7.6. The column was washed with 100 ml of this buffer followed by 100

ml 0.2 M NaCl, 100 ml 0.28 M NaCl, 100 ml 0.32 M NaCl, 100 ml 0.44 M NaCl, and 100 ml 1 M NaCl in 50 mM Tris/HCl pH 7.6 at a flow rate of 2 ml/min. The 0.32 M fraction contained the F_{420}-reducing hydrogenase activity and the polyferredoxin, as revealed subsequently.

The 0.32 M NaCl fraction (80 ml, 400 mg protein) was made to 1.4 M in $(NH_4)_2SO_4$ and loaded onto a Phenyl Sepharose HiLoad column (2.6 × 10 cm). The column was washed with 40 ml of 1.4 M $(NH_4)_2SO_4$ in 50 mM Tris/HCl, pH 7.6, followed by 40 ml 0.8 M $(NH_4)_2SO_4$, 40 ml 0.6 M $(NH_4)_2SO_4$, 50 ml 0.5 M $(NH_4)_2SO_4$, 50 ml 0.4 M $(NH_4)_2SO_4$, 50 ml 0.2 M $(NH_4)_2SO_4$, and 100 ml 50 mM Tris/HCl, pH 7.6. The 0.5 M fraction (25 ml, 20 mg protein) was brown in colour and the dominant protein had an apparent molecular weight of 44 kDa. The F_{420}-reducing hydrogenase was recovered in the fraction which eluted in the absence of $(NH_4)_2SO_4$.

The 0.5 M $(NH_4)_2SO_4$ fraction was desalted, concentrated by ultrafiltration (YM 30 membrane, cut-off 30 kDa), and applied to a hydroxylapatite column (1.6 × 15 cm) equilibrated with 30 mM potassium phosphate, pH 7. The column was washed with 40 ml of this buffer followed by 50 ml 100 mM potassium phosphate, pH 7, and by 50 ml 500 mM potassium phosphate, pH 7, at a flow rate of 2 ml/min. The 100 mM potassium phosphate eluate (20 ml, 10 mg protein) contained the polyferredoxin. It was further treated by ultrafiltration with Centricon 100 microconcentrators (cut-off 100 kDa). The filtrate contained the polyferredoxin (20 ml, 2 mg protein) which was then desalted on Centricon 30 microcentrators (cut-off 30 kDa). The desalted sample was chromatographed on a Mono Q 10/10 column equilibrated with 50 mM Tris/HCl, pH 7.6. The column was washed with 20 ml of this buffer and protein was eluted with a linear NaCl gradient (0.3–0.6 M). The protein was concentrated and desalted with Centricon 30 microconcentrators and stored at 4°C in 50 mM Tris/HCl, pH 7.6, plus 2 mM DTT.

Before determination of non-heme iron and acid-labile sulfur the protein solution was incubated with 1% Chelex 100 (Bio-Rad Laboratories, München, Germany) for 20 min.

3. RESULTS

The purification procedure described in Materials and Methods resulted in 1–2 mg of a dark-brown protein. In the following it is shown to be the *mvhB* gene product and to be a polyferredoxin.

3.1. *Apparent molecular weight and N-terminal amino acid sequence*

SDS-PAGE of the purified protein revealed only one band, of apparent molecular weight 44 kDa, which was, however, broad and fuzzy (Fig. 1), a property frequently observed for proteins with high cysteine content. Alkylation of the protein with iodoacetamide prior to SDS-PAGE yielded two distinct bands of apparent molecular weights of 60 and 56 kDa.

The apparent molecular weight of the native protein, determined by gel filtration on Superose 12, amounted to 95 kDa.

The N-terminal amino acid sequence of the 60 kDa iodoacetamide alkylated protein was analyzed after transfer from SDS polyacrylamide gel onto Pro Blott PVDF membrane (Applied Biosystems, Weiterstad, Germany) as described in [12]. The method employed did not allow discrimination between *S*-carboxamidomethyl cysteine and glutamate. The decision for cysteine or for glutamate was made such as to best fit the

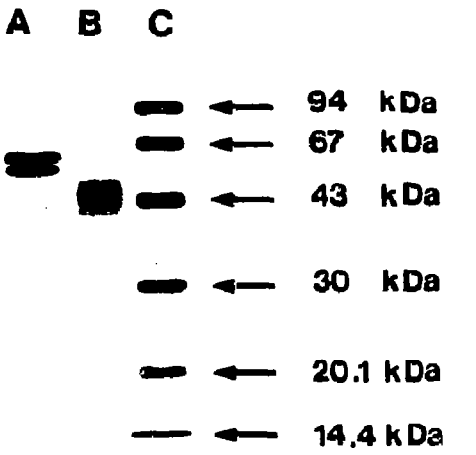

Fig. 1. Analysis of the purified polyferredoxin by SDS-PAGE. Separation was on 12% slab gels (8 × 7 cm) which were subsequently stained with Coomassie brillant blue R 250. (Lane A) Polyferredoxin (3 μg protein) alkylated with iodoacetamide as described in [11]. Prior to SDS-PAGE the protein was boiled in a solution containing 200 mM Tris/HCl, pH 8, 1% SDS and 13 mM DTT for 5 min. After boiling the solution was made to 100 mM in iodoacetamide and incubated for 30 min at 20°C; (lane B) polyferredoxin (3 μg protein). Prior to SDS-PAGE the protein was boiled in a solution containing 62 mM Tris/HCl, pH 6.8, 2% SDS and 16 mM DTT; (lane C) molecular weight standards from Pharmacia (Freiburg, Germany; electrophoresis calibration kit for low molecular weight proteins).

DNA sequence of the *mvhB* gene. Via this procedure the following sequence was obtained:

MIVVNKEDCIRCGACQGTCPTAAIEVTP

This sequence differs only in position 3 from the amino acid sequence deduced from the DNA sequence of the *mvhB* gene in *M. thermoautotrophicum* (strain H) [8] in which an isoleucine- rather than a valine triplet is found [8]. This small deviation might be caused by the fact that the polyferredoxin was isolated from the Marburg strain of *M. thermoautotrophicum*, whereas the DNA sequence of the gene was obtained for the ΔH strain of *M. thermoautotrophicum*. The DNAs of the two strains have been shown to hybridize only to 46% [13].

3.2. *Iron and acid-labile sulfur content*

The purified protein contained, per mg protein (quantitated by amino acid analysis as described in [14]), 1.2 μmol non-heme iron (analyzed by the method of Fish [15]) and 0.96 μmol acid-labile sulfur (determined as described by Cline [16]). Per mol, the protein thus contains 53 mol Fe and 42 mol acid-labile sulfur which is in agreement with the prediction that the *mvhB* gene product might contain as many as 12 [4Fe–4S] clusters.

3.3. *UV/visible spectrum*

The UV/visible spectrum exhibited the characteristics of typical clostridial ferredoxin spectra with absorption maxima at 280 nm and 390 nm and a shoulder at 308 nm (Fig. 2). The A_{390}/A_{280} ratio was 0.73. The extinction coefficient at 390 nm was calculated to be 170,000

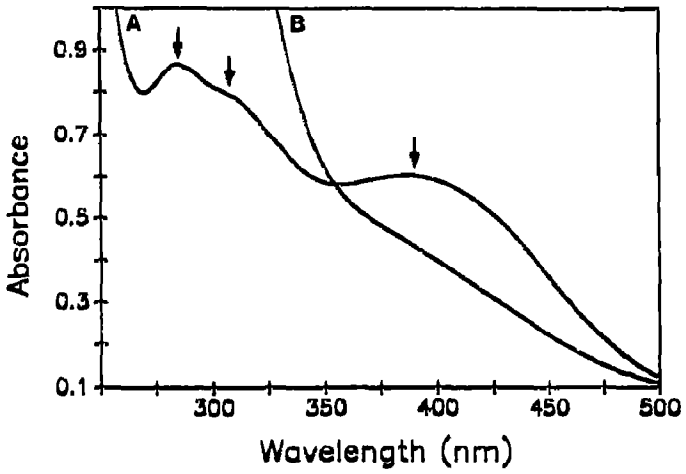

Fig. 2. Absorption spectrum of the purified polyferredoxin (95 μg protein in 0.6 ml 50 mM Tris/HCl, pH 7.6) (A) in the oxidized and (B) in the dithionite reduced state. The maxima at 280 and 390 nm and a shoulder at 308 nm are marked by arrows. The ratio A_{390}/A_{280} of the preparation was 0.73.

$M^{-1} \cdot cm^{-1}$. For a comparison, the ε_{390} of clostridial ferredoxin with 2 [4Fe–4S] clusters is 30,600 $M^{-1} \cdot cm^{-1}$ [17]. The ε_{390} is thus in agreement with the presence of 6 × 2 [4Fe-4S] clusters.

When the polyferredoxin was reduced with dithionite the absorbance at 390 nm decreased, a property characteristic for clostridial ferredoxins. The maximal $\Delta\varepsilon_{390}$ (ox-red) observed was 54,000 $M^{-1} \cdot cm^{-1}$.

3.4. *EPR properties*

In the oxidized form (as isolated) the polyferredoxin showed an EPR signal typical for a [3Fe–4S] cluster (Fig. 3). The intensity of this signal varied with the preparation and amounted to 5–50% of the polyferredoxin concentration. It could be enhanced several-fold by stirring the solution in air. Dithionite removed it and induced a broad signal in the $g = 2.1$ to 1.8 region (Fig. 3). In several preparations the intensity of this signal maximally amounted to 5-times the polyferredoxin concentration. Hence, only roughly half of the expected amount of [4Fe–4S] clusters was detected here.

3.5. *Reduction of the polyferredoxin with H_2 catalyzed by the MV-hydrogenase*

The rate of polyferredoxin reduction with H_2 at 120 kPa was measured at a concentration of 2 μM polyferredoxin (0.09 mg protein/ml) in the presence of 17 U MV-hydrogenase (isolated as in [7]; 870 U/mg protein assayed with benzylviologen as electron acceptor; 1 U = 2 μmol benzylviologen reduced per min at 60°C at pH 7.6) by following the decrease in absorbance at 390 nm using a $\Delta\varepsilon_{390}$ (ox–red) of 54,000 $M^{-1} \cdot cm^{-1}$. The specific rates observed were 15 nmol polyferredoxin reduced per min and mg hydrogenase protein at 60°C in 50 mM Tris/HCl, pH 7.6, corresponding to 90 nmol H_2 oxidized per min and mg protein assuming that 6 mol H_2 are required to reduce 1 mol of polyferredoxin. Different

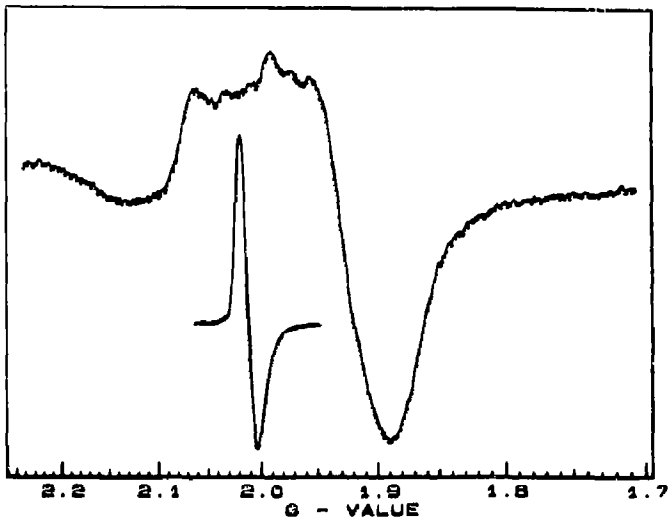

Fig. 3. EPR spectra of pure polyferredoxin (160 μg protein in 1 ml 50 mM Tris/HCl, pH 7.6). (Lower spectrum) sample as isolated; (Upper spectrum) sample after reduction with a few grains of dithionite. EPR conditions: microwave frequency, 9258.5 MHz; temperature, 13 K; microwave power incident to the cavity, 2 mW; modulation amplitude, 1.25 mT. The gain for the upper spectrum is 4.8-times that of the lower spectrum.

buffers and pH values (50 mM Tris/HCl pH 8.5, 50 mM CAPSO pH 9.0, 400 mM potassium phosphate pH 7.0) were tried in order to obtain higher specific activities but without success.

4. DISCUSSION

Methylviologen-reducing hydrogenase is considered to be involved in at least one step in CO_2 reduction with H_2 to methane, which in *M. thermoautotrophicum* cells proceeds at the specific rate of 3–4 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ [18]. This is the minimal specific rate at which the hydrogenase thus has to catalyze the oxidation of H_2. The finding that with polyferredoxin as electron acceptor the specific rate is only 0.09 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ makes it questionable whether this interesting protein is the physiological electron acceptor of the viologen-reducing hydrogenase although this cannot be excluded. For experimental reasons the specific activity had to be determined at very low polyferredoxin concentrations (2 μM) which were not saturating.

In *Clostridia*, ferredoxins function both as electron carriers and as iron storage proteins [19,20]. It can therefore also be envisaged that the polyferredoxin stores iron for the synthesis of the MV-hydrogenase which contains 19 Fe per mol [6].

It is interesting to note that sulfate-reducing bacteria contain a high molecular weight 65.5 kDa cytochrome *c* with 16 hemes [21,22], for which a function as electron acceptor for hydrogenase is discussed.

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