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The anabolic pyruvate oxidoreductase from *Methanococcus maripaludis*

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Abstract In autotrophic methanogens, pyruvate oxidoreductase (POR) plays a key role in the assimilation of CO_2 and the biosynthesis of organic carbon. This enzyme has been purified to homogeneity, and the genes from *Methanococcus maripaludis* were sequenced. The purified POR contained five polypeptides with molecular masses of 47, 33, 25, 21.5 and 13 kDa. The N-terminal sequences of four of the polypeptides had high similarity to the subunits commonly associated with this enzyme from other archaea. However, the 21.5-kDa polypeptide had not been previously observed in PORs. Nucleotide sequencing of the gene cluster encoding the POR revealed six open reading frames (*porABCDEF*). The genes *porABCD* corresponded to the subunits previously identified in PORs. On the basis of the N-terminal amino acid sequence, *porE* encoded the 21.5-kDa polypeptide and contained a high cysteinyl residue content and a motif indicative of a [Fe–S] cluster. *porF* also had a high sequence similarity to *porE*, a high cysteinyl residue content, and two [Fe–S] cluster motifs. Homologs to *porE* were also present in the genomic sequences of the autotrophic methanogens *Methanocaldococcus jannaschii* and *Methanothermobacter thermautotrophicus*. Based upon these results, it is proposed that PorE and PorF are components of a specialized system required to transfer low-potential electrons for pyruvate biosynthesis. Some biochemical properties of the purified methanococcal POR were also determined. This unstable enzyme was very sensitive to O_2 and demonstrated high activity with pyruvate, oxaloacetate, and α-ketobutyrate. Methyl viologen, rubredoxin, FMN, and FAD were readily reduced. Activity was also observed with spinach and clostridial ferredoxins and cytochrome *c*. Coenzyme F_{420} was not an electron acceptor for the purified enzyme.

Keywords *Methanococcus maripaludis* · Pyruvate oxidoreductase · Methanogen · Archaea

Abbreviations *CODH/ACS* Carbon monoxide dehydrogenase/acetyl-CoA synthase · *PBE* Polybuffer exchange · *POR* Pyruvate oxidoreductase

Introduction

Methanogenic bacteria are a major physiological group of archaea that obtain energy for growth by producing methane. Many typical hydrogenotrophic methanogens, such as *Methanococcus maripaludis*, are unable to oxidize complex organic substrates. The major methanogenic substrates are H_2+CO_2 or formate (Whitman 2001) and the major carbon sources are either CO_2 or acetate. During autotrophic CO_2 fixation, acetyl-CoA is formed by the modified Ljungdahl-Wood pathway (Ladapo and Whitman 1990; Shieh and Whitman 1988). When acetate is present, acetyl-CoA is formed by acetate thiokinase (Shieh and Whitman 1987). From acetyl-CoA, carbon enters the major biosynthetic pathways by reductive carboxylation, which is catalyzed by pyruvate oxidoreductase (POR) (Shieh and Whitman 1987). Therefore, this reaction is important for both autotrophic growth and heterotrophic growth on acetate.

POR activity has been detected in a wide range of microorganisms, including the archaea, bacteria, anaerobic protists, and fungi. The enzyme catalyzes a reversible reaction that has different physiological roles in these microorganisms (for reviews, see Adams and Kletzin 1996 and Charon et al. 1999). For many heterotrophic bacteria, pyruvate oxidation by POR provides electrons for nitrogen fixation and anaerobic respiration (Bosgusz et al. 1981). Pyruvate is also oxidized during acetate fermentation of sugars in homoacetogenic bacteria (Furdui and Ragsdale 2000). In autotrophic bacteria or bacteria that utilize acetate as a carbon source, POR biosynthesizes pyruvate.

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Because the reverse, biosynthetic reaction requires a strong reductant, it has been more difficult to demonstrate. The biosynthetic activity of the enzyme from the hydrogenotrophic bacterium *Hydrogenobacter thermophilus* was demonstrated with dithionite-reduced ferredoxin (Yoon et al. 1997). Similarly, in the presence of ferredoxin, carbon monoxide dehydrogenase and CO, POR from *Clostridium [Moorella] thermoaceticum* readily biosynthesizes pyruvate (Furdui and Ragsdale 2000). Lastly, low-potential ferredoxins reduced by the light-driven reactions of spinach chloroplasts or *Chlorobium* reaction centers drive pyruvate biosynthesis by *Chlorobium tepidum* POR (Yoon et al. 1999, 2001). However, with the exception of the light-driven reductions, these reactions are nonphysiological, and the mechanisms by which hydrogen-consuming lithotrophs generate low-potential electron donors for POR are not clearly understood.

In our previous studies on whole cells of methanococci, the oxidative activity of POR could not be demonstrated in vivo under normal growth conditions with H_2 (Yang et al. 1992). For instance, in an acetate auxotroph of *M. maripaludis*, the nutritional requirement for acetate could not be replaced by pyruvate. Similarly, the close relative *Methanococcus voltae* requires acetate for growth, and pyruvate will not substitute (Whitman et al. 1982). However, methanogenesis from pyruvate was detected in resting cells of both *M. maripaludis* and *M. voltae* in the absence of the physiological electron donors H_2 and formate (Yang et al. 1992). Thus, pyruvate was taken up and oxidized under at least some conditions. Pyruvate was also used as the sole energy and carbon source by mutants of *Methanosarcina barkeri* (Bock et al.1994; Rajagopal and LeGall 1994). These results suggest that the direction of catalysis by POR is strictly regulated in whole cells of methanogens. In methanococci, the oxidative activity of POR is readily demonstrated in cell-free extracts, but the biosynthetic activity is extremely labile. Incubation of extracts under N_2 gas for 1 h reduces the activity by 60% (Shieh and Whitman 1988). In addition, the physiological electron carrier is not certain. A ferredoxin purified from the closely related *Methanothermococcus thermolithotrophicus* was not reduced by POR (Hatchikian et al. 1989). However, the genomic sequence of *M. maripaludis* contains 18 ORFs annotated as ferredoxin or ferredoxin-related proteins (J. Leigh, University of Washington, personal communication), so it is possible that another ferredoxin may be coupled to this enzyme. In order to further characterize POR in methanococci, the enzyme was purified to homogeneity and the nucleotide sequence of its genes was determined.

Material and methods

Bacterial strains

M. maripaludis strain JJ (DSMZ 2067) was obtained from W.J. Jones (Jones et al. 1983). *Escherichia coli* strain Top 10 was obtained from Invitrogen (Carlsbad, Calif, USA).

Media, culture conditions, and preparation of cell extracts

M. maripaludis was grown with 275 kPa of H_2:CO_2 (80:20) at 37 °C in McN (mineral medium) or McC (complex medium minus vitamin solution) as described by Whitman et al. (1986). Large-scale cultures for enzyme purification were grown in a 400-l fermentor with mineral media (McN) and 0.5% sodium formate at 37 °C (Xing and Whitman 1992). Cells were harvested at the mid-stationary growth phase and resuspended in 10 ml of 25 mM potassium PIPES buffer, pH 6.8, with 0.5 mg DNase I per 10 g cellular wet weight. The suspension was frozen at –20 °C for 30 min to lyse the cells. Upon thawing in a water bath at room temperature, the supernatant was collected by centrifugation at 30,000×*g* at 4 °C for 30 min (Shieh and Whitman 1987). In some cases, the cell suspension was stored at –20 °C prior to the centrifugation.

E. coli strain Top 10 was grown at 37 °C on low-salt Luria-Bertaini medium as described by Invitrogen. For growth of cultures containing the plasmid pZErO-2 on liquid and solid medium, kanamycin (50 μg ml^{-1}) was added. Plasmids were transformed into *E. coli* strain Top 10 with a Gene Pulser (BioRad, Richmond, Calif., USA) at 200 W, 2.5 kV, and 25 mF with 0.2-mm-gap cuvettes.

Growth of both *M. maripaludis* and *E. coli* was monitored at 600 nm with a spectrophotometer (Spectronic 20, Bausch and Lomb).

Enzyme assays

The POR activities of chromatography fractions were determined anaerobically as pyruvate- and CoA-dependent methyl viologen reduction (Meinecke et al. 1989; Zeikus et al. 1977). The assay buffer contained 100 mM potassium Tricine, pH 8.6, with 20 mM pyruvate, 100 μM CoA, 10 mM methyl viologen, 5 mM $MgCl_2$, and 1 mM thiamine pyrophosphate. To monitor the effect of pH on POR, the following buffers were used: MES (100 mM, pH 5.5–6.5), HEPES (100 mM pH 7.0–7.5), Tricine (100 mM, pH 7.5–8.5), CHES (100 mM, pH 9.0–10.0), and CAPS (100 mM, pH 10.0–10.5). After flushing with N_2, a trace amount of dithionite was added until the buffer turned light blue. Assays were carried out at 37 °C under N_2 atmosphere. The increase of the absorbance at 578 nm was measured; the extinction coefficient for methyl viologen was 9.7 mM^{-1} cm^{-1} (Zeikus et al. 1977). For other electron acceptors, the activities were determined by the following extinction coefficients (mM^{-1} cm^{-1}): coenzyme F_{420}, ε_{420}=45.5 (Jones and Stadtman 1980); rubredoxin, ε_{491}=8.85 (Lovenberg and Walker 1978); ferredoxin, ε_{420}=17.3 (Uyeda and Rabinowitz 1971); FAD and FMN, ε_{450}=11 (Reeves et al. 1977); NAD^+ and $NADP^+$, ε_{340}=6.22 (Reeves et al. 1977); horse heart cytochrome *c*, ε_{550}=29.5 (Sigma); and vitamin K_1, ε_{326}=18.9 (Dunphy and Brodie 1971). To characterize the catalytic properties of the enzyme, activity was assayed as described above except that the assay buffer was 100 mM HEPES (N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid]), pH 7.5, at room temperature. Similar assays were used to determine the activities of formate dehydrogenase, hydrogenase, and CO dehydrogenase, except that pyruvate was replaced with 13 mM sodium formate, 100% of H_2 in the headspace, or 100% of CO in the head space, respectively. One unit of enzyme activity was defined as 1 μmol of methyl viologen reduced min^{-1}.

Protein concentration and molecular mass determinations

Protein concentrations were determined from the UV absorbance (Groves et al. 1968). As noted below, the Bio-Rad Protein Assay Reagent (Melville, N.Y., USA), based on Bradford's method using bovine serum albumin as the standard, was also used. The native molecular mass of POR was determined by both gradient native polyacrylamide gel electrophoresis (PAGE) as described below and gel filtration chromatography on Superose 6 (Pharmacia, LKB; Piscataway, N.J., USA) and Sephacryl S 150 (Sigma; St. Louis, Mo., USA). Molecular masses of subunits were determined by SDS-PAGE using the mini PROTEIN II system (Bio-Rad).

Specific activity of POR during growth

Cells were grown in a 10-l fermentor with McN or McN plus 10 mM acetate under 134 kPa H_2/CO_2 (80:20, v/v). When the culture OD_{660} reached 0.4, 0.6, 0.8, and 0.9, 200 ml of culture was collected in a nitrogen-flushed 1-l bottle. Cells were harvested by centrifugation in rubber-sealed centrifuge bottles at 10,000×*g* for 20 min at 4 °C. The centrifuge bottles had been previously equilibrated in an anaerobic chamber for 1 day prior to their use. POR activities and protein concentrations in the cell-free extracts were determined as described above.

Purification of POR

Unless specified differently, all procedures were carried out in a Coy anaerobic chamber at 2 °C using strictly anaerobic techniques. The basic buffer contained 20 mM potassium Tricine, pH 8.6 (at 25 °C), 5 mM $MgCl_2$, 0.1 mM TPP, 0.5 mM dithiothreitol, and 10% (v/v) glycerol. Unless specified otherwise, columns were washed with 1.5 bed volumes of buffer after loading the sample and eluted with gradients consisting of five bed volumes. Chromatography was done with an FPLC system (Pharmacia Biotech; Piscataway, New Jersey). Samples were centrifuged at 4 °C in rubber-sealed centrifuge tubes that had been allowed to stand in the anaerobic chamber for 1 day.

Streptomycin sulfate treatment

Cell extract was centrifuged again at 48,000×*g* for 30 min at 4 °C. The supernatant was diluted to a protein concentration of 15 mg/ml with basic buffer. A 5% (w/v) streptomycin sulfate solution in basic buffer (0.3 volumes) was added to the diluted protein solution. The supernatant was collected after centrifugation at 37,000×*g* for 10 min at 4 °C (Lehman 1989).

Ammonium sulfate treatment

A solution of saturated ammonium sulfate was slowly added to the supernatant until 45% saturation was obtained. The well-mixed solution was incubated at 2 °C for 20 min. The supernatant was collected by centrifugation at 30,000×*g* for 20 min at 4 °C.

Phenyl Sepharose CL-4B chromatography

The phenyl Sepharose CL-4B column (2.6×34 cm; Pharmacia Biotech) was equilibrated with 1 M ammonium sulfate in basic buffer. After loading the ammonium sulfate supernatant, the column was washed with equilibrating buffer, and protein was eluted with a decreasing gradient of 1–0 M ammonium sulfate at a flow rate of 19 cm/h. The column was then washed with basic buffer followed by an increasing gradient of ethylene glycol from 0 to 50% (v/v) in basic buffer. The low molecular weight and the high molecular weigh forms of POR eluted at 0.06 M and 0.16 M ammonium sulfate, respectively.

Q Sepharose fast-flow chromatography

Active fractions from the previous column were pooled, diluted with three volumes of basic buffer, and loaded onto a Q Sepharose fast-flow column (2.6×34 cm; Pharmacia Biotech) equilibrated with basic buffer at a flow rate of 60 cm/h. The column was washed with 0.2 M NaCl in the basic buffer. POR was then eluted with a gradient of 0.2–0.7 M NaCl in the basic buffer. The low molecular weight and the high molecular weight forms of POR eluted at 0.41 M and 0.45 M NaCl, respectively. Active fractions were pooled and dialyzed overnight against 2 l of basic buffer.

Blue agarose chromatography

The dialyzed pool was applied to a blue agarose column (1.6× 12.5 cm; Cibacron Blue 3GA, Sigma) that had been equilibrated with basic buffer at a flow rate of 18 cm/h. Chromatography was carried out with a linearly increasing gradient starting with basic buffer and ending with 1 M NaCl in basic buffer. The low molecular weight and the high molecular weight forms of POR both eluted at 0.22 M NaCl. The active pool was dialyzed overnight against 2 l of basic buffer.

PBE-94 chromatography

The dialyzed pool was loaded onto a polybuffer exchange (PBE) column (1.0×15 cm; Pharmacia Biotech) equilibrated with basic buffer at a flow rate of 15 cm/ml. POR was eluted with an increasing gradient of NaCl in basic buffer and 1 M NaCl in basic buffer. The low molecular weight and high molecular weight forms of POR eluted at 0.45 M and 0.50 M NaCl, respectively. The active fractions were pooled and concentrated to 0.2 ml by centrifugal ultrafiltration at 3,000×*g* (Microsep, molecular mass cut-off 10 kDa; Filtron; Northborough, Mass., USA).

Superose 6 chromatography

For some purifications, PBE-94 chromatography was followed by Superose 6 chromatography. A Superose 6 column (1.6×30 cm; Pharmacia Biotech) was equilibrated with basic buffer containing 1 M NaCl, 20 µM FAD, and 10% ethylene glycol. The concentrated PBE pool was loaded onto the column and eluted with the equilibrating buffer. The low molecular weight and the high molecular weight forms of POR eluted at M_r of 222,000 and 2,075,000, respectively. The active fractions were pooled and stored anaerobically at –20 °C.

Gel electrophoresis

Electrophoresis was carried out on the Mini-PROTEAN II (Bio-Rad). The protein solution was dialyzed against distilled water at 4 °C overnight and concentrated on a Speed Vac to 10µl. Both 8% native and SDS-(14%) polyacrylamide gels were run in Tris-glycine buffer, pH 8.3, at 150 V. For the 4–25% gradient native gel, electrophoresis was done in Tris-borate-EDTA buffer at 160 V for at least 16 h at 4 °C. Gels were stained with 0.25% Coomassie brilliant blue in 25% isopropanol and 10% acetic acid and photographed. To quantify the staining intensity of the bands, the image from the negative was scanned and analyzed with ImageQuant v.1.2 for Macintosh software (Molecular Dynamics, Sunnyvale, Calif., USA). For 2-dimensional PAGE, a 4–25% gradient native gel was run for the first dimension. After electrophoresis, the protein bands were visualized by staining with Coomassie brilliant blue. The stained band was cut from the gel and incubated with SDS-PAGE sample buffer for 15 min. The gel slice was loaded onto the top of an SDS-polyacrylamide gel for electrophoresis in the second dimension. Proteins in the gel were detected by silver staining (Garfin 1990).

N-terminal amino acid sequencing

POR subunits were separated by SDS-PAGE, and the polypeptides were transferred to the polyvinylidene difluoride (PVDF) membrane by electroblotting in a mini Trans-Blot cell (Bio-Rad). The blotting buffer was 25 mM Tris-base, 192 mM glycine, 10% (v/v) methanol, pH 8.3, and the blotting time was 45 min at 90 V. The polypeptides were sequenced on a 477A Protein Sequencer (Applied Biosystems) at the Molecular Genetics Facility, University of Georgia.

UV-visible spectrum

Purified enzyme, 150 µg in 1 ml, was dialyzed against basic buffer containing 0.5 M NaCl overnight at 4 °C. Dialysis buffer was used as the blank for the spectrum. The UV-visible spectrum was measured on a Shimadzu UV 2101-PC UV-VIS scanning spectrophotometer at room temperature.

Purification of coenzyme F_{420}

All purification steps were carried out in the dark following the general protocol of Eirich et al. (1978). About 50 g wet weight of *M. maripaludis* cells were suspended in 100 ml of H_2O. The suspension was heated at 90 °C for 15 min. After centrifugation at 6,000×*g* for 10 min at room temperature, the supernatant was loaded on to a DEAE cellulose column (1.6×35 cm, Sigma). Coenzyme F_{420} was eluted with a linear gradient of five bed volumes of ammonium bicarbonate (0–0.6 M). The fractions with a high absorbance at 420 nm were pooled and concentrated in a flash evaporator at 80 °C. The concentrated pool was loaded onto a Sephadex G 10–120 column (1.5×76 cm, Sigma) at a flow rate of 5 cm/ml. Coenzyme F_{420} was eluted with 0.1 M ammonium bicarbonate. Coenzyme F_{420}-containing fractions were then loaded onto a DEAE Sephadex A 25 column (1.6×10 cm; Pharmacia Biotech) and eluted with a gradient of five bed volumes of ammonium bicarbonate (0–1.3 M) at a flow rate of 10 cm/h. Coenzyme F_{420} was highly purified; the A_{245}/A_{420} ratio was 1.5. The ratio of the pure coenzyme was 1.0 (Eirich et al. 1978).

Probe construction, Southern hybridization and screening of clones

A probe, MJPOR, containing a portion of the *Methanococcus jannaschii* genes for POR was constructed from the plasmid AMJHM83 (American Type Culture Collection, Md). The cloned DNA was excised from the plasmid using *Sac*I and *Xba*I and radiolabelled using a modified random nucleotide-primed synthesis method (Lehman 1989). *M. maripaludis* genomic DNA was isolated by a modification of the procedure of Saito and Miura (1963) as described previously (Gardner and Whitman 1999). Prior to Southern hybridization, the *M. maripaludis* genomic DNA was digested with *Hin*dIII, *Eco*RI or both enzymes at the same time. For Southern hybridization, the digested DNA fragments (15 µg) were electrophoresed, transferred to a Magnacharge nylon membrane (MSI, Mass., USA). Prehybridization and hybridization with probe MJPOR were done at 28 °C for 4 and 10 h, respectively, in 2×SSC. The membrane was washed twice for 15 min at 38 °C in 0.5×SSC. Following a 10-h exposure to the phosphor screen, hybridization was visualized using the Phosphorimager:SI (Molecular Dynamics, Sunnyvale, Calif., USA) and quantified using the ImageQuant v. 1.2 software. The hybridization revealed a 1.4-kb *Hin*dIII fragment, a 6.1-kb *Eco*RI fragment, and a 1.4-kb fragment from the double digest.

Because of the low stringency of the hybridization of the MJPOR probe to the *M. maripaludis* DNA, the *Hin*dIII fragment was isolated first and used as a probe for the 6.1-kb *Eco*RI fragment. First, the genomic DNA was digested with *Hin*dIII and electrophoresed, and the 1.4-kb region of the gel was excised. The DNA was purified with the Wizard Plus Miniprep system (Promega, Wis., USA) and ligated into *Hin*dIII-digested pZErO cloning vector (Invitrogen). The ligation mixture was then transformed into *E. coli* Top 10 cells (Invitrogen). Plasmid DNA purified from kanamycin-resistant isolates were screened by Southern hybridization with probe MJPOR using the same conditions as described above. The radiolabelled probe MmPORαβ was then constructed from the plasmid containing the *Hin*dIII fragment by PCR using primers PorMj83-5′ (5′-AGTCATCCTCTGCCTTTTCA) and PorMj83-3; (5′GATTACAAGACCTTCAGGG). The amplification solution consisted of 50 pg DNA template, 25 pmol of each primer, 200 mM cNTP (dTTP, dCTP, dGTP), 20 nM of [α-^{32}P]dATP (3,000 Ci/nmol), and 1.5 U of Taq polymerase (Promega) along with the reaction buffer supplied with the enzyme. After denaturation of the DNA at 95 °C for 5 min, PCR was carried out as follows: 25 cycles of 94 °C, 1 min; 50 °C, 1 min; and 72 °C, 1 min. The final PCR amplicon was purified from unincorporated nucleotides with a NucTrap Probe (Stratagene, LaJolla, Calif., USA) purification column. The 6.1-kb *Eco*RI fragment was then isolated from a partial library of *Eco*RI-digested genomic DNA in the same manner as the *Hin*dIII fragment except that the MmPORαβ probe was used.

Sequencing and analyses

Plasmids from *E.coli* Top 10 isolates hybridizing to the probes were obtained from a 5-ml culture of LB broth containing kanamycin and purified (Qiagen spin miniprep, Calif.). The genomic inserts were sequenced with M13 reverse primer (5′-CAGGAAACAGCTATGAC) and the M13 forward primer (5′-GTAAAACGACGGCCAG), which were complementary to flanking regions of pZErO-2 (Invitrogen). Through primer walking, the DNA was sequenced on both strands. Sequencing was done on an Applied Biosystems automated sequencer (PE Applied Biosystems, Calif., USA) at the Molecular Genetics Facility at the University of Georgia. The sequences were assembled using the program Sequencher (Gene Codes, Mich., USA).

For the phylogenetic analyses, nucleic acid sequences were obtained from the GenBank-EMBL database and translated into polypeptide sequences. Similarity searches were carried out using the BLAST and FASTA programs from GCG (Genetics Computer Group, Madison, Wis., USA) and the TIGR website. Sequences of homologs from other euryarchaeotes of about the same size were chosen for further analysis. Unaltered sequences, subsets of sequences, and conserved regions within the sequences were used for multiple alignments using PILEUP and BOXSHADE programs of GCG. Phylogenetic analyses were done using PHYLIP v. 3.57 (Felsenstein 1989). Evolutionary distances were determined with PROTDIST, and the neighbor-joining and Fitch-Margoliash dendrograms were generated with NEIGHBOR and FITCH, respectively. Parsimony analyses were done with PROTPARS. The SEQBOOT program was used to calculate bootstrap values based upon 100 replicate trees. The Genbank accession number for the *M. maripaludis* 6.1-kbp *Eco*RI fragment containing the POR genes is AF230199.

Results

POR activity in cell-free extracts

In order to purify the enzyme, the stability of the methanococcal POR in cell extracts was determined. Activity was rapidly inactivated by O_2. Following incubation in air at 2 °C for 40 min, about 60% of enzyme activity was lost. However, no loss of activity of the partially purified enzyme was detected after 3 weeks of storage under anaerobic conditions at –20 °C (data not shown). Following dialysis, methanococcal POR activity was very unstable in the absence of glycerol or ethylene glycol (data not shown). TPP and $MgCl_2$ also helped maintain activity. No activity was lost after dialysis of extract in a basic buffer containing 20 mM potassium Tricine, pH 8.6, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM TPP, and 10% glycerol, and anaerobic storage for two weeks at 2 °C. The POR specific activity of cell extracts was unaffected by the growth phase. Following growth in mineral medium to optical densities of 0.4–0.9, the specific activities were 2.2–2.4 U (mg of protein)$^{-1}$. Under these growth conditions, cultures are in the exponential growth phase at optical densities below

Table 1 Purification of the methanococcal POR. The starting material was 100 g wet weight of cells

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Recovery (%)
Cell extract	4880	3610	1.3	(100)
Streptomycin supernatant	4510	3010	1.5	93
$(NH_4)_2SO_2$ precipitation	3670	1100	3.3	75
Phenyl Sepharose	860	100	8.4	18
Q Sepharose	610	43	14	13
Blue agarose	480	12	38	10
Poly buffer exchange-94	400	5.0	79	8

0.4. From optical densities of 0.4–0.8, cultures are in the linear phase during which growth appears to depend upon the rate of transfer of H_2 gas to the medium. At an optical density of 0.9, cultures are in early stationary phase. In a similar experiment, the addition of 10 mM sodium acetate to the culture medium did not affect the specific activity (data not shown).

Purification and stability of POR

In a typical purification, POR was purified 60-fold with an 8% yield (Table 1). Further purification by chromatography on Superose 6 was required to obtain electrophoretically homogeneous enzyme (Fig. 1, lane 1). Integration of the Coomassie-stained SDS-polyacrylamide gel indicated a purity of about 97% for these preparations (Fig. 1, lane 3). However, the yield at the Superose 6 step was variable and depended critically on the concentration of NaCl. In a systematic study of the affect of buffer upon the recovery of the partially purified enzyme after Superose 6 chromatography, yields of greater than 30% were only obtained with 0.4 M or greater concentrations of NaCl plus FAD. The recovery at this step was further improved to about 45% with buffer containing 1 M NaCl, 20 µM FAD, and 10% ethylene glycol (data not shown). The low total recovery was due in part to the extreme lability of this enzyme, and specific activities of the purified enzyme varied from 36–155 U (mg of protein)$^{-1}$. Purifications which were done in a shorter amount of time yielded product with a higher specific activity, and the variable recovery was not correlated with the subunit composition. After purification, activity was further lost in a matter of days upon storage at 4 °C even under anaerobic conditions. In general, activity was lost when both the protein and salt concentrations were low. Similarly, the PORs from many organisms are unstable upon purification, and the PORs from *Clostridium acetobutylicum* as well as other methanogens are also sensitive to dilution (Bock et al. 1996; Meinecke et al. 1989; Tersteegen et al. 1997).

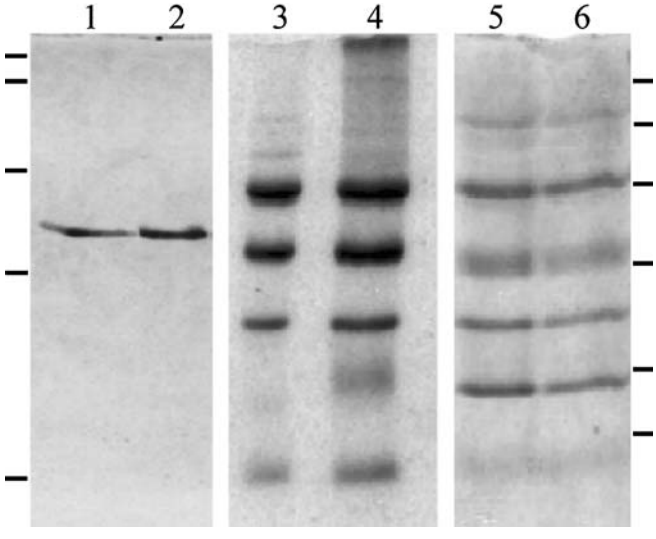

Fig. 1 Native and SDS-PAGE of the purified low molecular weight pyruvate oxidoreductase (POR) and high molecular weight PORs following Superose 6 chromatography in basic Tricine buffer containing 1.0 M NaCl, 20 µM FAD, and 10% ethylene glycol. *Lanes 1 and 2*: 4–30% gradient PAGE of the undenatured low molecular weight POR (*lane 1*) and the high molecular weight POR (*lane 2*) stained with Coomassie blue. Molecular mass markers of 669 kDa, 440 kDa, 232 kDa, 158 kDa and 67 kDa are indicated by *bars* on the *left*. *Lanes 3–6*: SDS-PAGE of the POR. The molecular masses of 97.4 kDa, 66.2 kDa, 45 kDa, 31 kDa, 21.5 kDa, and 14.4 kDa are indicated by the *bars* on the *right*. The low molecular weight POR (*lane 3*) and high molecular weight POR (*lane 4*) were stained with Coomassie blue. *Lanes 5 and 6*: After gradient PAGE, the low molecular weight POR (*lane 5*) and the high molecular weight POR (*lane 6*) were excised and run on SDS-PAGE. These lanes were silver stained

In one purification, a second, overlapping peak of POR activity was observed during the phenyl-Sepharose chromatography in addition to the activity purified above. Because of the low yields of the purification, it was important to determine whether this second activity was another enzyme or a form of the original POR. To this end, the second activity was purified (data not shown). After chromatography on PBE-94, the specific activity and yield of the new form were 68 U (mg of protein)$^{-1}$ and 6%, respectively, which were comparable to the values obtained for the original form during this purification. By gradient native PAGE, the second form also appeared to be nearly homogeneous and possessed the same molecular weight as the first form purified (Fig. 1, lane 2).

Molecular weight and composition of POR

Although the molecular mass of both forms were near 180 kDa by gradient native PAGE, the native molecular masses of the new (high molecular weight) and original (low

molecular weight) forms were 2,100 kDa and 200 kDa, respectively, by Superose 6 chromatography. In addition, the native molecular weight of the low molecular weight form was determined by chromatography on Sephacryl S150 as 185,000. Presumably, the high molecular weight form dissociated during electrophoresis on native gels, resulting in the lower molecular weight by that method. At this point, it was not possible to determine which form was physiologically important. For instance, the low molecular weight form could have formed by dissociation of a high molecular weight form during the purification. Alternatively, the high molecular weight form could be formed by aggregation of the low molecular weight form.

In initial studies of the low molecular weight enzyme, four polypeptides were observed following SDS-PAGE (Fig. 1, lane 3). These polypeptides corresponded to the subunits commonly found in the multisubunit PORs: α (47 kDa), β (33 kDa), γ (25 kDa) and δ (13 kDa). The subunit stoichiometry determined by integration of the Coomassie-stained gel was 1:1.08:0.90:1.32 for the α:β:γ:δ subunits, or close to the expected values of 1:1:1:1. In addition to the four subunits observed in the low molecular weight POR, the high molecular weight POR contained a fifth polypeptide (21.5 kDa) that migrated as a diffuse band (Fig. 1, lane 4). On the basis of integration of the Coomassie-stained gel, the stoichiometry of this polypeptide was comparable with that of the other subunits, i.e. 0.98 relative to the α subunit. This band was present in all SDS-polyacrylamide gels of this preparation. Moreover, careful examination of gels of the low molecular weight POR also revealed small amounts of this polypeptide, with a stoichiometry of about 0.10 compared to the α subunit. Although some high molecular weight material at the top and near 68 kDa was also present on some gels (Fig. 1, lanes 4–6), this material was only observed sporadically and appeared to have resulted from incomplete denaturation of the enzyme.

These results suggested that the composition of the high molecular weight POR differed from that of the low molecular weight POR by the presence of an additional polypeptide. However, this conclusion proved not to be true. To confirm the subunit compositions of the PORs, the high and low molecular weight enzymes were first electrophoresed by native gradient PAGE. After staining, the gel band was excised, treated with SDS sample buffer, and separated by SDS-PAGE. Following this second electrophoresis, the fifth polypeptide (21.5 kDa) was observed in both forms of the POR (Fig. 1, lanes 5, 6). In an attempt to explain the different results for the low molecular weight POR, the two protocols were examined in detail. A major difference was that the samples were not heated in preparation for SDS-PAGE during the second procedure. Therefore, the low molecular weight POR was prepared for SDS-PAGE without heating, and the 21.5 kDa polypeptide was also visible (data not shown). Therefore, both the low and high molecular weight forms of POR appeared to contain five polypeptides. For whatever reason, the fifth polypeptide in the low molecular weight form appeared to be heat labile. Possibly, the low molecular weight form was contaminated by small amounts of heat-activated protease.

To confirm the identities of the POR subunits, the N-terminal amino acid sequences of the polypeptides were determined. For the low molecular weight POR, the sequences were: 47-kDa polypeptide: L-E-V-K-V-I-T-G-T-L-A-A-L-E-A-A-K-L-A-D-V-L-X-I-A-A-Y; 33-kDa polypeptide: L-G-S-Q-F-P-R-E-L-G-F-A-P-G-H; 25-kDa polypeptide: M-K-E-V-R-F-H-G-R-G-G-Q-G-A-V-T-A-A-Q-I-L-A-K-A-A-F-Y-D; and 13-kDa polypeptide: V-N-T-G-T-I-I-Y-E-P-G-S. These sequences showed similarity to the N-terminal sequences of the POR subunits from other methanogens (Tersteegen et al. 1997) and confirmed the homology of the methanococcal enzyme. For the high molecular weight POR, the sequences for the 47-kDa and 25-kDa subunits were found to be identical with those of the low molecular weight POR. Therefore, these preparations were forms of the same enzyme. In addition, the N-terminal sequence of the 21.5-kDa polypeptide of the high molecular weight form was M-K-K-V-M-M-V-N-E-A-X-D-N-X-G-D-X-V-K-S.

The UV-visible spectrum of the purified enzyme under anaerobic conditions was determined. The enzyme possessed a broad absorbance between 350 and 500 nm, which was consistent with the presence of nonheme Fe. Assuming a molecular weight of 190,000, the extinction coefficient of methanococcal POR at 280 nm and 390 nm was 2.8×10^5 M^{-1} cm^{-1} and 7.4×10^4 M^{-1} cm^{-1}, respectively, when the protein concentration was determined by Bradford's method with bovine serum albumin as the standard.

Catalytic properties

Because the physiological electron donor for the methanococcal POR was not identified (see below), the catalytic properties of the nonphysiological, oxidative reaction were determined using artificial electron acceptors. These properties are expected to be somewhat different from those of the biosynthetic reaction determined with the physiological electron carrier. Nevertheless, they are of interest for comparative purposes. The specific activity of the purified enzyme was affected by the reaction temperature and pH. At 37 °C, maximal activity was obtained at pH 7.3 (data not shown). Comparable activities were obtained in both HEPES and Tricine buffers at this pH. At pH 7.3, the temperature optimum was 60 °C, and the activity detected at 60 °C was five times the activity detected at 37 °C (data not shown). The Arrhenius plot was also biphasic. Over the 25–40 °C range, the activation energy was 84.3 kJ/mol. Over the 40–60 °C range, the activation energy was 45.5 kJ/mol. This latter value was close to that found for the POR from *Methanosarcina barkeri* (Bock et al. 1996).

The purified enzyme had a broad substrate specificity. In the presence of CoA, it oxidized pyruvate, oxaloacetate, α-ketobutyrate, and indol-3 pyruvate with specific activities of 7.4, 6.5, 3.6, and 0.3 U mg^{-1}, respectively. These values were lower than those obtained with freshly isolated enzyme because of the rapid loss in activity upon

Table 2 Electron acceptors for the *M. maripaludis* POR. The low molecular weight form of POR was used after the PBE column. The activities with the electron donors were determined by the extinction coefficients as described in Materials and methods

Electron acceptor	Concentration	Specific activity (U mg^{-1})
Methyl viologen	5 mM	9.05
Coenzyme F_{420}	26 µM	<0.004
Clostridial rubredoxin	20 µM	4.02
Clostridial ferredoxin	2.5 µM	0.22
Spinach ferredoxin	8.3 µM	0.22
FAD	0.5 mM	1.27
FMN	0.5 mM	3.17
NAD^+	5 mM	<0.15
$NADP^+$	5 mM	<0.15
Cytochrome *c*	0.1 mM	0.48
Vitamin K1	1.1 µM	<0.10

storage. In contrast, the oxidation of α-ketoglutarate, α-ketoisovalerate, α-ketoisocaproate, and phenyl pyruvate were less than 0.05 U mg^{-1}, which was the limit of detection for this assay. The apparent K_ms for pyruvate, oxaloacetate, and α-ketobutyrate were 115, 264, and 205 µM, respectively. The apparent V_{max}s were 7.7, 5.0, and 3.6 U (mg of protein)$^{-1}$, respectively. Assuming a molecular weight of 190,000, the k_{cat} values were 27, 18, and 13 s^{-1}, respectively. With 20 mM pyruvate, at 37 °C, pH 7.3, the apparent K_m and V_{max} for CoA were 5.8 µM and 8.1 U (mg of protein)$^{-1}$, respectively. Substrate inhibition by CoA is common and has been observed with POR from a number of sources (Inui et al. 1989; Kerscher and Oesterhelt 1981; Meinecke et al. 1989; Uyeda and Rabinowitz 1971; Williams et al. 1987). However, it was not observed with the methanococcal enzyme with CoA concentrations up to 100 µM.

The methanococcal POR reduced clostridial rubredoxin, clostridial and spinach ferredoxin, cytochrome *c*, FMN, and FAD (Table 2). No activity was detected with NAD^+, $NADP^+$, and vitamin K1. The low activities with the ferredoxins were consistent with the inability of a ferredoxin purified from *Methanothermococcus thermolithotrophicus* to couple to POR from that organism (Hatchikian et al. 1989). Because the *M. maripaludis* genome contains 18 ORFs, annotated as ferredoxin or ferredoxin-related proteins (J. Leigh, University of Washington, personal communication), it is possible that another ferredoxin may couple to POR. Rubredoxin was also an electron acceptor for the enzyme from *Chlorobium tepidum* (Yoon et al. 1999). However, given its electropositive midpoint potential, it was unlikely to be a physiological electron carrier of the methanococcal POR.

Because flavins were readily reduced, it seemed possible that FMN or FAD could be the physiological electron carrier. Moreover, FAD stabilized the enzyme during chromatography and inhibited the reduction of methyl viologen (see below), suggesting that the POR contained a flavin binding site. However, the apparent K_ms, V_{max}s, and k_{cat}s for FMN and FAD were 4.6 and 5.0 µM, 2.5 and 2.1 U (mg of protein)$^{-1}$, and 8.7 and 7.4 s^{-1}, respectively. These K_m values were somewhat higher than is common for many flavoproteins but is within the range found for enzymes that use free flavins as substrates (Filisetti et al. 2003). However, the catalytic efficiencies or k_{cat}/K_m values were calculated to be 2.4–3.2×10^4 min^{-1} M^{-1}, which were about two orders of magnitude lower than observed for the likely physiological electron carriers of other PORs (Furdui and Ragsdale 2000, Yoon et al. 2001). Therefore, it is unlikely that flavins were the physiological electron carrier of the methanococcal POR. However, it still is possible that flavins play an important regulatory or structural role in the enzyme.

Table 3 Activity of the purified POR and cell extracts with coenzyme F_{420}. Activities were determined in 100 mM HEPES buffer, pH 7.5, at 37 °C. *FDH* Formate dehydrogenase, *MV* methyl viologen, –, not determined

	POR specific activity (U/mg of protein)		FDH[b] specific activity (U/mg of protein)	
	MV	coenzyme F_{420}	MV	coenzyme F_{420}
Cell extract	1.43	0.61	184	48
Dialyzed cell extract	1.92	0.45	178	73
Purified enzyme[a]	4.95	<0.0004	–	–
+ FAD	0.16	<0.007	–	–

[a]The POR was the low molecular weight form after the PBE column

Although coenzyme F_{420} was reduced in the dialyzed cell extract with pyruvate and CoA and has been proposed to be the physiological electron donor (Zeikus et al. 1977), it was not utilized as an electron acceptor by purified POR (Table 3). No decrease of the absorbance at 420 nm was observed after 30 min of incubation of reaction mixtures at 37 °C in the dark. To test the possibility that a flavin was required that had been removed during purification, the enzyme was preincubated with 10 µM FAD or FMN at 37 °C for 5 min prior to the assay. However, this preincubation inhibited subsequent methyl viologen reduction and did not activate coenzyme F_{420} reduction. As a control for the quality of the coenzyme, the preparation of coenzyme F_{420} was reduced by the formate dehydrogenase activity in cell extracts, and the ratio of activities with F_{420}/methyl viologen, 0.26, was in the normal range, 0.01–0.42 (Table 3). Thus, failure of the methanococcal POR to use coenzyme F_{420} as an electron donor was not due to modification of the coenzyme that may have occurred in purification (Baron and Ferry 1989; Muth et al. 1987; Nelson et al. 1984; Schauer and Ferry 1983). In conclusion, like the enzyme from *Methanosarcina* (Bock et al. 1996), the methanococcal POR is not coenzyme F_{420}-dependent.

Inhibitors

The activity of the purified enzyme was inhibited by O_2 and dithionite, but only slightly or not at all by glyoxylate, nitrite, CO or potential physiological effectors. Compared

with the enzyme in the cell extract, the purified enzyme was more sensitive to O_2. The half-life was 5.2 min when the purified enzyme was exposed to air in an ice bath. In contrast, about 40% of the activity in the cell extract remained after 40 min in air at 2 °C. After inactivation of the purified enzyme by oxygen, activity was not restored by replacing the oxygen with nitrogen and adding 10 µM dithionite, a quantity sufficient to produce a light blue color in the assay solution containing methyl viologen. In addition to oxygen, the purified enzyme was also inactivated by sodium dithionite, which is sometimes added to purifications to protect O_2-sensitive enzymes. Enzyme activity was lost during incubation in buffer containing dithionite, and the loss of activity was dithionite-dependent. With 5 mM dithionite, the half-life was 7 h at 2 °C, with about 90% of the original activity being lost after 24 h. The enzyme was also weakly inhibited by high concentrations of glyoxylate and nitrite. In the presence of 20 mM glyoxylate, about 40% of the original activity was lost. However, 2 mM glyoxylate inhibited only 6%. Thus, the glyoxylate inhibition was not as potent as found for the enzyme from other sources. In cell-free extracts of *Clostridium kluyveri*, the K_i of the glyoxylate was 61 µM (Thauer et al. 1970). In cell-free extracts of *M. thermautotrophicus*, 2 mM glyoxylate was required for the 67% inhibition (Zeikus et al. 1977). With 20 mM sodium nitrite, about 29% of the original activity was lost. In contrast, the K_i for nitrite by the *Pyrococcus furiosus* enzyme was 11 mM (Blamey and Adams 1993). Unlike the pyrococcal enzyme, the methanococcal POR was not inhibited by CO. Potential affectors such as ATP, ADP, AMP, cAMP, GTP, GDP, GMP, NAD^+, NADH, and glyceraldehyde 3-phosphate, at concentrations of 2 mM, did not affect the activity of purified POR.

Identification of the *M. maripaludis por* cluster

The *M. jannaschii* genomic sequence possessed two clusters of ORFs, annotated as ferredoxin-dependent oxidoreductases (Bult et al. 1996). One of these was identified as POR based upon similarity to the N-terminal sequences from the *M. maripaludis* polypeptides. A probe for the *M. maripaludis* genes, MJPOR, was then constructed from the plasmid AMJHM83 (Fig. 2). This plasmid contained a 1.7-kb insert that included *porC* in its entirety and portions of *porB* and *porD* from *M. jannaschii*. Southern hybridization identified 6.1-kb and 1.4-kb fragments from *Eco*R1- and *Hin*dIII-digested *M. maripaludis* genomic DNA, respectively. Double digestion of genomic DNA confirmed that the *Hin*dIII fragment was internal to the *Eco*RI fragment. A partial library was created using *Hin*dIII-digested *M. maripaludis* genomic DNA and screened by hybridization to MJPOR. Upon sequencing, the clone containing the 1.4-kb *Hin*dIII insert DNA was found to include sequences homologous to a region within *porC* from the *M. jannaschii por* gene cluster. The *Hin*dIII fragment from *M. maripaludis* was then used as a probe to isolate a clone possessing the 6.1-kb *Eco*RI fragment.

Analysis of the nucleotide sequence of the 6.1-kb *Eco*RI fragment revealed nine complete and one partial ORFs, four of which (*porABCD*) had sequence similarity to POR genes identified from *P. furiosus* and *M. jannaschii* (Fig. 2) (Adams and Kletzin 1996; Bult et al. 1996). The homologous genes from *M. thermautotrophicus* appeared to contain a fusion of *porA* and *porB* from these other organisms. The percent similarity of the amino acid sequences of the genes in the *M. maripaludis por* gene cluster to *P. furiosus, M. jannaschii*, and *M. thermautotrophicus* genes was in the range of 48–79% (Fig. 2). *porA, porB, porC*, and *porD* were 527, 254, 1160, and 890 bp, respectively, and encoded for polypeptides of approximately 19.6, 9.4, 42.1, and 33.2 kDa, respectively. These estimated molecular masses corresponded favorably to the M_rs of the polypeptides from the purified enzyme. Additionally, the deduced N-terminal sequences of *porABCD* corresponded to the N-terminal sequences of the *M. maripaludis* POR α, β, γ, and δ subunits. Further analysis of the *porABCD* sequences revealed potential ribosomal binding sites for all four ORFs, motifs characteristic of conserved [4Fe–4S] clusters in both *porA* and *porB*, and motifs suggestive of a characteristic [4Fe–4S] cluster and TPP-binding site in *porD*. These motifs were very similar to those found in the *P. furiosus* and *M. jannaschii* genes. Moreover, all the motifs required for coenzyme and

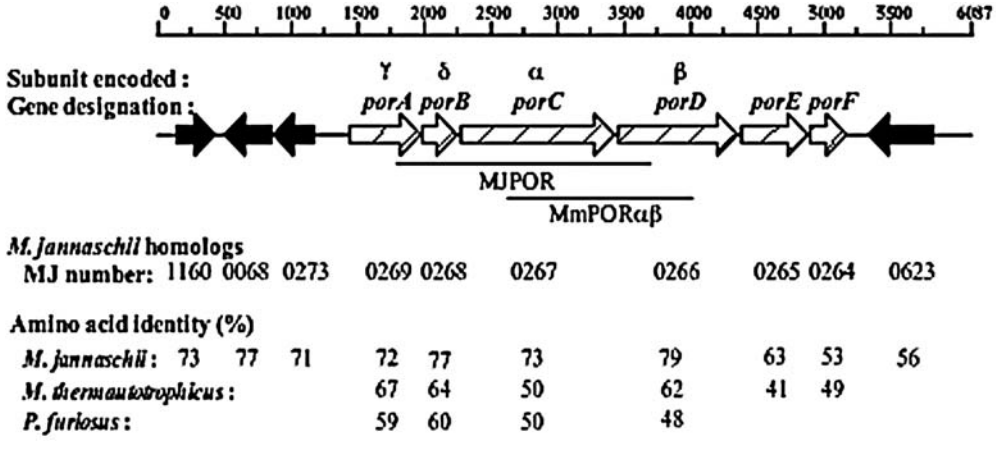

Fig. 2 Sequence of the 6.1-kb *Eco*RI fragment containing the *por* genes from *M. maripaludis* genomic DNA. MJPOR represents the location of the *M. jannaschii* clone that was used to isolate the original *M. maripaludis Hin*dIII fragment MmPORαβ. Hybridization to the *Hin*dIII fragment was used to isolate this *Eco*RI fragment encoding the entire gene cluster

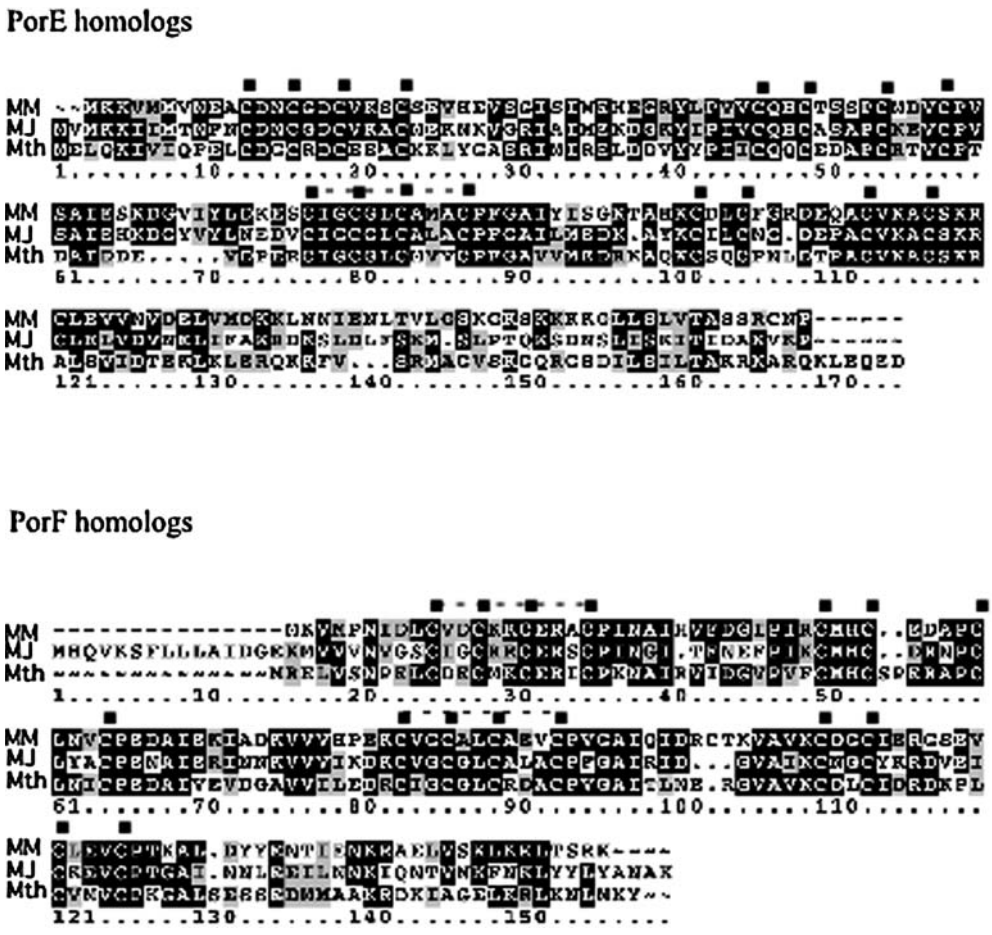

Fig. 3 Structural features of the PorE and PorF homologs from *Methanococcus maripaludis* (MM), *Methanocaldococcus jannaschii* (MJ), and *Methanothermobacter thermautotrophicus* (Mth). Conserved cysteinyl residues are indicated by ■. *Dotted lines* denote the location of the consensus sequence for the binding sites of [4Fe–4S] clusters

[Fe–S] cluster binding that were identified in the crystal structure of the *Desulfovibrio africanus* POR were found (Chabrière et al. 1999).

Analysis of *porEF*

Immediately downstream of *porD* were two ORFs, referred to here as *porE* and *porF*. These ORFs were expected to encode polypeptides of 167 and 138 amino acids, 18 and 17 of which were cysteinyl residues, respectively (Fig. 3). They were named PorE and PorF to avoid confusion with the previously named subunits of the catabolic PORs. One motif indicative of [4Fe–4S] clusters was present in *porE*, while two motifs were present in *porF*, suggesting a possible function in electron transfer. Moreover, these proteins have sequence similarity to the DmsB subunit of the dimethylsulfoxide reductase, which has four [4Fe–4S] clusters (Rothery and Weiner 1991). The estimated molecular mass of PorE was 18.5 kDa, which was similar to the actual molecular mass of the 21.5-kDa polypeptide that copurified with the POR. In addition, the N-terminal sequence of this polypepetide was identical to that of the translation product of *porE*.

Homologs with high similarity to PorE and PorF were also present immediately downstream of the *por* gene clusters in the genomic sequences of the autotrophic methanogens *M. jannaschii* (MJ0265 and MJ0264, respectively) and *M. thermautotrophicus* (MTH1737 and MTH1736, respectively) but not in the gene clusters encoding PORs from other archaea such as *Archaeoglobus fulgidus* and *P. furiosus*. Other homologous cysteine-rich ORFs were also found immediately downstream to the CODH/ACS gene cluster in *M. maripaludis, M. jannaschii,* and *M. thermautotrophicus*. In phylogenetic analyses, the homologs associated with *porE*, *porF* and CODH/ACS gene clusters grouped together (Fig. 4). For instance, the *porF* homologs clustered together with high bootstrap support when analyzed by neighbor-joining, Fitch-Margoliash, and parsimony methods in both an alignment of the nearly complete sequences and an alignment of only the most conserved regions. Although the bootstrap support was somewhat lower, the CODH/ACS gene cluster was also found by all three algorithms (Fig. 4). While the *porE* homologs formed a cluster with low bootstrap support in the neighbor-joining and Fitch-Margoliash analyses (Fig. 4), MTH1737 formed a deep branch associated with the homologs from the CODH/ACS gene cluster in the most parsimonious trees. The somewhat different placement by different algorithms was not surprising given the small sizes of these sequences and the small number of informative positions. Together, the phylogenetic similarities and locations on

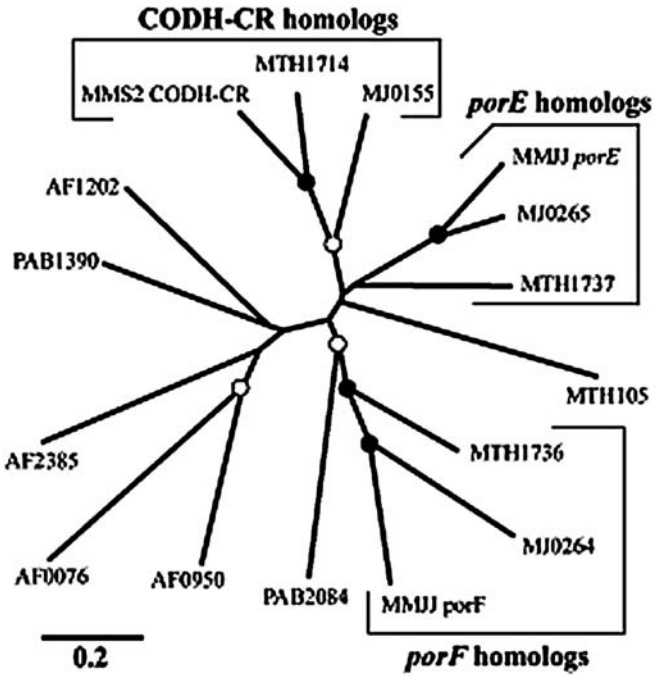

Fig. 4 Phylogenetic relationships of the PorE homologs associated with the POR and CODH/ACS gene clusters of the hydrogenotrophic methanogens *M. maripaludis* (MMJJ for strain JJ and MMS2 for strain S2), *M. thermautotrophicus* (MTH) and *M. jannaschii* (MJ). For comparison, additional homologous ORFs of about the same size and high sequence similarity were included from the euryarchaeotes *Archaeoglobus fulgidus* (AF) and *Pyrococcus abyssii* (PAB). The dendrogram was based upon an alignment of conserved positions and was generated by the Fitch-Margoliash algorithm. With the exceptions discussed in the text, it was largely congruent with the dendrograms generated by neighbor-joining and parsimony (not shown). Bootstrap values for all three dendrograms were also nearly identical and indicated by the symbols on the branch-points: values >88% are indicated by (●), values >70% are indicated by (○). *Scale bar* 0.2 expected amino acid substitutions per site

the genomes suggested that these genes have similar functions in the different hydrogenotrophic methanogens and that they were conserved within these organisms.

Other homologs with high sequence similarity formed separate clades. *M. jannaschii* had two additional homologs that were much larger in size (data not shown). MJ1193 was greater than twice the size of *porE* and was a subunit of the methyl viologen-reducing hydrogenase. It was also homologous to the *M. thermautotrophicus* ORF MTH1133. Similarly, the homolog MJ1303 contained greater than 300 amino acid residues more than *porE*. The *M. thermautotrophicus* genome sequence contained one additional homolog, MTH0105, that appeared to represent a fusion to a subunit of glutamate synthase. *A. fulgidus* possessed four homologs (AF1202, AF0076, AF0950, AF2385) of about the same size as *porE*, but they were not linked to the gene clusters encoding the POR or CODH/ACS and did not group with the methanococcal ORFs in phylogenetic analyses (Fig. 4). AF1202 was found in the genome near a subunit of formate dehydrogenase and was similar to a homolog in *Pyrococcus abyssii* PAB1390 (Fig. 4), but there was no evidence for the function of the other ORFs. In addition, *A. fulgidus* contained AF0175, a homolog that was larger than *porE* and near a DMSO reductase subunit in the genome (data not shown). Lastly, additional homologs were found in the *P. abyssii* and *P. horikoshi* genomic sequences. The ORFs PAB2084and PH0893 (in *P. horikoshi*) were very similar and appeared next to a subunit of formaldehyde dehydrogenase. In the neighbor-joining and the Fitch-Margoliash analyses, these ORFs clustered with *porF* with low bootstrap support (Fig. 4). However, in the parsimony analyses, they clustered with the *Archaeoglobus* ORFs AF0076 and AF0950 (data not shown). In addition to these homologs with high similarity, there were a large number of homologs of low similarity from a variety of archaea and bacteria. These ORFs were typically annotated as formate hydrogenlyase subunit 2 (HycB), a subunit of dimethylsulfoxide reductase DmsB, homologs of YsaA and HydN, glutamate synthase, or ferredoxin.

Discussion

For hydrogenotrophic methanogens, the biosynthesis of pyruvate is bioenergetically unfavorable under the conditions of methanogenesis in the environment. For instance, at 10^{-4} atm of H_2, which is typical for many habitats of methanogens, the E′ of the H_2/H^+ couple is only –296 mV (Zinder 1993), or far less than the midpoint potential of the acetyl-CoA/pyruvate couple of about –500 mV (calculated from Thauer et al. 1977). Thus, the ΔG for the reduction of acetyl-CoA + CO_2 under these conditions would be about +39 kJ/mol. This change in free energy is so positive that the reaction would be expected to remain unfavorable in the presence of any physiologically likely concentration of substrates. For instance, the pool sizes of HS-CoA and acetyl-CoA in *Methanothermobacter marburgensis* (formerly *M. thermautotrophicus* strain Marburg) are 40 µM and 52 µM, respectively (Rühlemann et al. 1985). Given CO_2 and H_2 concentrations of 10^{-1} atm and 10^{-4} atm, respectively, the cellular concentration of pyruvate at equilibrium would be about 20 nM. This concentration is far below the physiological concentration and implies that an additional energy source is necessary for pyruvate biosynthesis by the hydrogenotrophic methanogens.

At the beginning of these studies, two types of energy sources for pyruvate biosynthesis seemed possible. The first type consisted of sources intrinsic to the pyruvate oxidoreductase reaction. Thus, pyruvate biosynthesis might require hydrolysis of a high-energy substrate such as ATP to drive the reaction. However, the catalytic properties of the purified POR from *M. maripaludis* are similar to those of the catabolic enzyme from *Pyrococcus* and other sources (for a review, see Adams and Kletzin 1996). If ATP or some other substrate was required for reduction of acetyl-CoA + CO_2, then the hydrolysis product would be expected to be required for the oxidation of pyruvate. No evidence was obtained for additional substrates in the ox-

idative reaction. Therefore, this possibility is unlikely. Similarly, the enzyme from the hydrogenotrophic *M. thermautotrophicus* also appears to be a typical POR (Tersteegen et al. 1997). Another intrinsic mechanism might be coupling pyruvate biosynthesis directly to the proton-motive force. In this case, the POR would be expected to be membrane-associated. Although this possibility can not be easily eliminated, the POR from *M. maripaludis* appeared in the soluble fraction and was not an intrinsic membrane protein.

In the absence of clear evidence for an intrinsic coupling to an energy source, extrinsic possibilities need to be considered. In this type of coupling, cells could utilize an additional energy source to generate a strong reductant for the POR so that pyruvate biosynthesis would be favorable. As precedents for this model, the catabolic POR of *Clostridium* [*Moorella*] *thermoaceticum* biosynthesized pyruvate in vitro when the enzyme was supplied with a strong reductant by coupling to the carbon monoxide dehydrogenase and CO oxidation (Furdui and Ragsdale 2000). Although this reaction is possible in vitro, it is not plausible physiologically because high concentrations of CO required are not present. Similarly, the biosynthetic reaction of the *Chlorobium tepidum* POR requires special low-potential ferredoxins (Yoon et al. 2001). Presumably, these ferredoxins would be reduced by light-driven reactions in this organism. For methanococci, a detailed physiological model for coupling pyruvate biosynthesis to an energy source is not known. However, an attractive possibility is that the energy in the proton-motive force would be utilized to drive the unfavorable reduction of a very electronegative electron acceptor by H_2. *M. maripaludis* contains two homologs of the membrane-bound ech hydrogenase found in *M. thermautotrophicus* and *Methanosarcina barkeri* (W.B. Whitman, unpublished results, and J. Leigh, University of Washington, personal communication; Künkel et al. 1998; Meuer et al. 1999). This enzyme system contains components homologous to the mitochondrial complex I and is believed to couple H_2-oxidation to the proton-motive force (Albracht Hedderich 2000). In *Methanosarcina*, mutants in this enzyme complex lose the ability to biosynthesize pyruvate (Meuer et al. 2002), providing direct evidence for its role in generating a strong reductant for POR in that organism.

The discovery of a novel cysteinyl-rich polypeptide in the anabolic POR of methanococci suggests that this protein could be a component of a specialized system required to transfer low-potential electrons for pyruvate biosynthesis. The evidence in support of this proposal is five-fold.(1) Because PorE copurified with the POR, it must be capable of binding to this enzyme. (2) PorE and its homologs possess the structural features common to polyferredoxins and other electron carriers, including [Fe–S] motifs and a high cysteinyl content. Therefore, the limited structural information that is available is consistent with this function. (3) Although homologs of these proteins have not been well characterized and their midpoint potentials have not been determined, they typically play some role as electron carriers. Annotated as formate hydrogenlyase subunit 2, a domain of the large subunit of glutamate synthase, or ferredoxin, they appear to function over a broad range of midpoint potentials more electronegative than quinones (Böck and Sawers 1996; Vanoni and Curti 1999). In *E. coli*, the formate hydrogenlyase subunit 2 is also proposed to couple with the ech hydrogenase (Sauter et al. 1992), a function very similar to the one proposed here for PorE. For the function proposed here, the midpoint potential of the methanococcal proteins would have to be near –500 mV. (4) The distribution of homologs of PorE in other methanococci as well as *Methanothermobacter* support its presumed role in the anabolic reaction. Its absence from organisms in which the POR is primarily catabolic, such as *Pyrococcus*, is consistent with this interpretation. However, the absence of homologs associated with the *Archaeoglobus* POR genes and the *M. barkeri* enzyme suggest that these proteins may be a specialized adaptation of the hydrogenotrophic methanogens and not necessarily a general mechanism for all anabolic PORs in archaea. (5) The presence of a homolog associated with the operon encoding the CODH/ACS provides further support for the proposed function. Like the POR, this enzyme system is also be expected to require a low-potential electron donor to generate the carbonyl group of acetyl-CoA from CO_2. Although PorF was not found associated with the purified POR, it seems likely that it also would play an important role in this process for many of the same reasons. Presumably, this polypeptide is more weakly associated with the enzyme and is removed during the purification. However, further experiments will be necessary to test the role of these proteins in the POR as well as CODH/ACS reactions.

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