Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 340 (2006) 76–82
BBRC
www.elsevier.com/locate/ybbrc

Anabolic five subunit-type pyruvate:ferredoxin oxidoreductase from *Hydrogenobacter thermophilus* TK-6

Takeshi Ikeda, Toshihiro Ochiai, Susumu Morita, Ayako Nishiyama, Eio Yamada, Hiroyuki Arai, Masaharu Ishii *, Yasuo Igarashi

Department of Biotechnology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

Received 8 November 2005
Available online 6 December 2005

Abstract

The thermophilic, obligately chemolithoautotrophic hydrogen-oxidizing bacterium, *Hydrogenobacter thermophilus* TK-6, assimilates carbon dioxide via the reductive tricarboxylic acid cycle. A gene cluster, *porEDABG*, encoding pyruvate:ferredoxin oxidoreductase (POR), which plays a key role in this cycle, was cloned and sequenced. The nucleotide sequence and the gene organization were similar to those of the five subunit-type 2-oxoglutarate:ferredoxin oxidoreductase from this strain, although the anabolic POR had been previously reported to consist of four subunits. A small protein (8 kDa) encoded by *porE*, which had not been detected in the previous work, was identified in the purified recombinant POR expressed in *Escherichia coli*, indicating that the enzyme is also a five-subunit type. Incorporation of PorE in the wild-type POR enzyme was confirmed by immunological analysis. PorA, PorB, PorG, and PorE were similar to the α, β, γ, and δ subunits of the four subunit-type 2-oxoacid oxidoreductases, respectively, and had conserved specific motifs. PorD had no specific motifs but was essential for the expression of the active enzyme.

Keywords: *Hydrogenobacter thermophilus*; Pyruvate:ferredoxin oxidoreductase; 2-Oxoacid oxidoreductase; 2-Oxoglutarate:ferredoxin oxidoreductase; Reductive tricarboxylic acid cycle; Thermophile

Pyruvate:ferredoxin oxidoreductase (POR) is the enzyme that catalyzes thiamine pyrophosphate (TPP)-dependent oxidative decarboxylation of pyruvate to form acetyl-CoA and CO_2. In contrast to pyruvate dehydrogenase, which is a generally distributed enzyme catalyzing the same reaction, POR can also catalyze the reverse reaction, i.e., the reductive carboxylation of acetyl-CoA provided that a sufficiently low-potential electron donor is available. The reverse reaction is an important step for some autotrophs because it serves to assimilate CO_2 into cell carbon.

POR is distributed among archaea, bacteria, and anaerobic protozoa, and is a member of the 2-oxoacid oxidoreductase (OR) family, which catalyzes the oxidative decarboxylation of 2-oxoacids to their acyl- or aryl-CoA derivatives. Most ORs from hyperthermophilic archaea and bacteria, such as *Pyrococcus furiosus* and *Thermotoga maritima*, consist of four distinct subunits (α, β, γ, and δ) [1]. In contrast, mesophilic bacterial ORs, such as those from *Desulfovibrio africanus* [2] and *Moorella thermoacetica* (f. *Clostridium thermoaceticum*) [3], are homodimers of a single large subunit, which is a fusion of the four subunits in the α–γ–δ–β arrangement. ORs from *Sulfolobus tokodaii* (f. *Sulfolobus* sp. strain 7) [4] and *Halobacterium halobium* [5] are two-subunit types. The large subunit is a γ–α fusion, and the small subunit corresponds to the β subunit of the four subunit-type enzymes. No δ subunit-like domain exists in this type of enzyme. The four subunit-type enzyme has been proposed to be the common ancestor that underwent gene rearrangement and fusion to generate single and two subunit-type enzymes [4,6,7].

* Corresponding author. Fax: +81 3 5841 5272.
E-mail address: amishii@mail.ecc.u-tokyo.ac.jp (M. Ishii).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.11.155

Hydrogenobacter thermophilus TK-6 is a facultatively aerobic, thermophilic, obligately chemolithoautotrophic hydrogen-oxidizing bacterium [8,9]. Phylogenetic analyses of 16S ribosomal RNA sequences have shown that the genus *Hydrogenobacter* is closely related to the genus *Aquifex* and that the *Hydrogenobacter–Aquifex* branch is the deepest branching order in the domain Bacteria [10,11]. It has been reported that *Hydrogenobacter* and *Aquifex* assimilate carbon dioxide via the reductive tricarboxylic acid (RTCA) cycle [12,13]. In this cycle, POR catalyzes reductive carboxylation of acetyl-CoA using a low-potential electron carrier such as a ferredoxin as the electron donor. POR was purified from *H. thermophilus* [14] and shown to catalyze the reductive carboxylation using a reduced ferredoxin purified from the bacterium as the electron donor [15]. The purified POR was reported to consist of four distinct α, β, γ, and δ subunits with apparent molecular masses of 46, 31.5, 29, and 24.5 kDa, respectively [14]. Another OR-family enzyme, 2-oxoglutarate:ferredoxin oxidoreductase (OGOR), is also involved in the RTCA cycle. *H. thermophilus* has two types of OGOR enzymes designated Kor and For. Kor is an archaeal-like two subunit-type enzyme encoded by *korAB* [16,17]. For is a novel five subunit-type enzyme encoded by *forDABGE*. The *kor* and *for* genes are located adjacent to each other and are divergently transcribed [18]. In this study, we cloned the gene cluster encoding the anabolic POR from *H. thermophilus* and found that it had a close relationship with the *for* gene cluster. We reexamined the quaternary structure of POR and found that the enzyme also has the novel five subunit-type structure.

Materials and methods

Bacterial strains and growth conditions. H. thermophilus strain TK-6 (IAM 12695, DSM 6534) was cultivated anaerobically with nitrate as a terminal electron acceptor as previously described [19]. *Escherichia coli* JM109 was used as a host for derivatives of the pUC vectors. *E. coli* TH2 was used as a host for the enforcement cloning pKF3 vector (TaKaRa). *E. coli* strains were grown in tryptic soy broth or Luria–Bertani medium at 37 °C. When necessary, 100 μg ml^{-1} ampicillin or 12.5 μg ml^{-1} of chloramphenicol was added to the medium.

Cloning and DNA sequencing. Colony and Southern hybridizations were performed using Hybond-N nylon membranes (Amersham Biosciences) and a DIG DNA labeling and detection kit (Roche Diagnostics). Nucleotide sequences were determined on an ABI 377 DNA sequencer (Applied Biosystems) using a BigDye terminator cycle sequence kit (Applied Biosystems) for dideoxy chain-termination reactions.

On the basis of the N-terminal amino acid sequence of the POR β subunit determined by Yoon et al. [14], a pair of degenerate primers (5′-TAYGARATHAAYGARGC-3′ and 5′-TCNACTATYTCYTTNGG-3′) was synthesized to amplify a fragment corresponding to the 5′-terminus of the *porB* gene, encoding the β subunit, by PCR using the chromosomal DNA of *H. thermophilus* as a template. The resultant 50-bp PCR fragment was ligated with pUC119 and sequenced. With another degenerate primer (5′-GARATHAAYGARGCTCTTAGAG-3′) based on the nucleotide sequence of the 50-bp PCR fragment and an LA PCR in vitro cloning kit (TaKaRa), the 0.4-kb partial *porB* gene was amplified by PCR and used as a probe for cloning. A 3.4-kbp *Sph*I fragment of the genomic DNA of *H. thermophilus*, hybridized with the probe by Southern hybridization. A gene library constructed by insertion of *Sph*I fragments into pUC118 was screened by colony hybridization, and two positive colonies were obtained out of 360 clones. The resultant plasmid containing the partial *porD* and complete *porABG* genes was designated pSM201. For further investigation of the upstream region, a 1.1-kbp *Sph*I–*Bam*HI digest of pSM201 was used as a probe. A 2.2-kbp *Pst*I fragment of the genomic DNA of *H. thermophilus* was hybridized with this probe by Southern hybridization. A gene library constructed by insertion of *Pst*I fragments into pKF3 was screened by colony hybridization, and 10 positive colonies were obtained out of 180 clones. The resultant plasmid containing the complete *porED* and partial *porA* genes was designated pSM301. The nucleotide sequence reported in this paper was deposited in the DDBJ nucleotide sequence database under Accession No. AB042412.

Enzyme assay and activity staining. The enzyme activity of POR was determined spectrophotometrically by following pyruvate- and CoA-dependent reduction of methyl viologen. The standard assay mixture contained 20 mM pyruvate, 0.5 mM CoA, 5 mM methyl viologen, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.5 mM TPP in 100 mM HEPES buffer (pH 8.0 at 20 °C). Assays were carried out at 70 °C under an argon atmosphere. The increase in absorbance at 578 nm was measured on a Hitachi U-3210 spectrophotometer equipped with a thermoelectric cell holder and a temperature controller. The reduction of methyl viologen was calculated using an extinction coefficient of 9.8 mM^{-1} cm^{-1}. One unit of enzyme activity was defined as the reduction of 1 μmol of methyl viologen min^{-1}. Activity staining on a non-denaturing gel was performed as previously described [14].

Construction of expression plasmids for POR. The *porEDABG* genes were amplified by PCR from the chromosomal DNA of *H. thermophilus* with a pair of primers, Eh-F (5′-AATAAAAGCTTATAAATTCCTTTTTAGGAGGC-3′), which introduced a *Hin*dIII site (underlined) before the initiation codon of *porE*, and Ge-R (5′-CC*GAATTC*TTTACTCAGTTAGC-3′), which introduced an *Eco*RI site (in italics) after the stop codon of *porG*. The obtained PCR product was digested with *Hin*dIII and *Eco*RI, and ligated with the *Hin*dIII–*Eco*RI digested pUC19. The nucleotide sequence of the inserted fragment was confirmed on both strands. The resultant plasmid was designated pUC-POR. Other expression plasmids, pUC-PdE and pUC-PdED, were constructed with other sets of primers Dh-F (5′-CATGCAAGCTTGTAAAACTTTTTAAAGGAGGTTT-3′) and Ge-R, and Ah-F (5′-AGTAAAAGCTTGTAAACCCTTTGTAAGGAG-3′) and Ge-R, respectively, in the same way. pUC-PdD was constructed by insertion of two fragments amplified with Eh-F and Ex-R (5′-TTTAA**CTCGAG**TTACTTTTCCGCATG-3′), and Ax-F (5′-AATGA**CTCGAG**TCATGCCACTGGC -3′) and Ge-R (see Fig. 1B). *Hin*dIII, *Eco*RI, and *Xho*I sites on the oligonucleotides are designated by underlined, italic, and bold letters, respectively.

Heterologous expression and purification of the recombinant POR. Cell extract of *E. coli* JM109 harboring pUC-POR was prepared after cultivation in tryptic soy broth supplemented with 0.5 mM $FeSO_4$, 1 mM IPTG, and 100 μg ml^{-1} of ampicillin under microaerobic conditions as previously described [17] and transferred into a vial in an anaerobic chamber (Coy Laboratory Products). After the vial was sealed with a rubber septum and an aluminum cap, the vial was heat-treated at 70 °C for 10 min. The heat-denatured proteins were removed by centrifugation at 30,000*g* for 20 min. Chromatography was performed with a BioLogic DuoFlow system (Bio-Rad) in the anaerobic chamber at room temperature. The buffer used throughout the purification procedure contained 1 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM dithionite, 0.1 mM TPP, 10% (v/v) glycerol, and 0.02% (v/v) Triton X-100. Heat-treated cell extract was loaded onto a Q Sepharose high performance column (Amersham Biosciences; 2.6 × 10 cm) equilibrated with 20 mM Tris–HCl buffer (pH 8.0). Proteins were eluted with a linear gradient from 0 to 1.0 M NaCl at a flow rate of 4 ml min^{-1}. The active fractions were pooled and loaded onto a CHT ceramic hydroxyapatite column (Bio-Rad; 1.6 × 10.5 cm) equilibrated with 4 mM potassium phosphate (pH 6.8). Proteins were eluted with a linear gradient from 4 to 400 mM potassium phosphate (pH 6.8) at a flow rate of 4 ml min^{-1}.

Protein assay. Protein concentrations were determined using an *RC DC* protein assay kit (Bio-Rad) with bovine serum albumin as the standard.

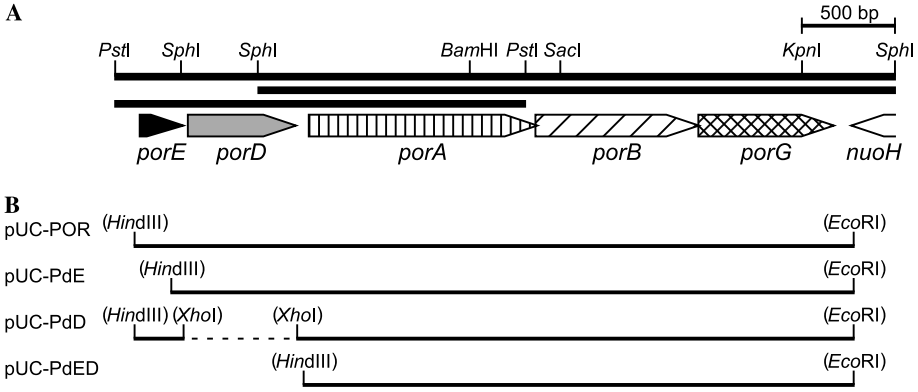

Fig. 1. (A) Physical map of the POR genes in the *H. thermophilus* genome. The arrow boxes indicate the size and orientation of the transcription of the genes. The relative positions of the cloned *Pst*I (2166 bp) and *Sph*I (3359 bp) fragments are indicated as bars above the arrow boxes. The deduced amino acid sequence of the partial *nuoH* gene was 75.3% identical (in 97 aa) to the sequence of *A. aeolicus* AAC07299 (*nuoH1* encoding a putative NADH dehydrogenase I chain H). (B) Bars indicate the fragments used for heterologous expression of the POR genes in *E. coli*. Restriction sites in parentheses were generated by PCR with synthetic oligonucleotide primers.

N-terminal amino acid sequencing. Subunits of the purified recombinant POR were separated by SDS–PAGE and blotted onto a Sequi-Blot PVDF membrane (Bio-Rad) with a semidry electroblotting system (HorizBlot AE-6677; Atto). N-terminal amino acid sequences were determined on a Procise 491 protein sequencer (Applied Biosystems).

Native molecular mass determination. The native molecular mass of the recombinant POR was estimated by gel filtration chromatography on a Superdex 200 column (Amersham Biosciences) as previously described [14], except that the concentration of Triton X-100 in the elution buffer was reduced from 0.1 to 0.02% (v/v) because its UV absorption overlaps that of proteins and interferes with the detection of proteins in column effluents. A Bio-Rad gel filtration standard was used as the calibration standard.

Western blot analysis. A 15-aa peptide (MYYVAEVINDECSKY) that corresponds to the N-terminal amino acid sequence of PorE was synthesized. The synthetic peptide was conjugated with keyhole limpet hemocyanin and used to immunize a rabbit to prepare the anti-PorE polyclonal antibody. Cytoplasmic proteins from the *H. thermophilus* cells were separated by non-denaturing PAGE and blotted onto a Sequi-Blot PVDF membrane as described above. Immunostaining was performed using a Konica immunostaining HRP-1000 kit as previously described [19].

Results

Cloning of the genes encoding POR

On the basis of the N-terminal amino acid sequences of the POR subunits [14], 3359-bp *Sph*I and 2166-bp *Pst*I fragments were cloned from the chromosomal DNA of *H. thermophilus*. Five complete open reading frames (ORFs) designated *porEDABG* were identified in the cloned 4111-bp region (Fig. 1A). Each ORF is preceded by a typical Shine–Dalgarno sequence [20]. A partial ORF, *nuoH*, which encodes a putative subunit of NADH dehydrogenase, is located downstream of the *por* genes in the opposite orientation.

The N-termini of the deduced amino acid sequences of the *porDABG* genes correspond to the reported N-terminal amino acid sequences of the δ, α, β, and γ subunits, respectively; however, they are not completely identical probably because of errors in the previous amino acid sequencing. The *porDABG* genes are 570, 1203, 861, and 714 bp in length, respectively, and encode polypeptides with molecular masses of 21.3, 44.3, 32.3, and 26.1 kDa, respectively. These estimated molecular masses correspond to the apparent values of the polypeptides from the previously purified enzyme. PorA, PorB, and PorG are similar to the α, β, and γ subunits of the four subunit-type PORs, respectively. They have 28.1–37.5% and 23.8–30.9% amino acid identity with those of *P. furiosus* POR and *T. maritima* POR, respectively. PorD is not similar to the δ subunit of the other four subunit-type PORs. On the contrary, *porE*, which is located upstream of *porDABG*, encodes a protein similar to the δ subunit. The deduced PorE protein is smaller (8.9 kDa) but has 37.0% and 34.1% amino acid identity with the δ subunit of *P. furiosus* POR (12.0 kDa) and *T. maritima* POR (11.3 kDa), respectively. PorD and PorE are similar to ForD and ForE of *H. thermophilus* with 64.3% and 69.6% of the amino acids being identical, respectively. ForD and ForE are subunits of the For enzyme, which is a novel five subunit-type OGOR [18]. The deduced sequences of the *porABG* genes are also highly similar to those of the *forABG* genes (Fig. 2). Although *H. thermophilus* POR had been previously reported to consist of four distinct subunits [14], these results suggested that the enzyme is a five-subunit type as is the case of For. Consistent with this suggestion, all of the motifs required for cofactor binding that were identified in the crystal structure of the *Desulfovibrio africanus* POR [21] were found in the deduced amino acid sequence of PorEDABG; PorB contains a conserved TPP-binding motif and seven cysteine residues, four of which comprise an atypical iron–sulfur cluster binding motif. PorE contains eight conserved cysteine residues that comprise two ferredoxin-like iron–sulfur cluster binding motifs.

Heterologous expression of the por genes in E. coli

The pUC19-derivative plasmids designated pUC-POR, pUC-PdE, pUC-PdD, and pUC-PdED were constructed for the heterologous expression of the recombinant POR

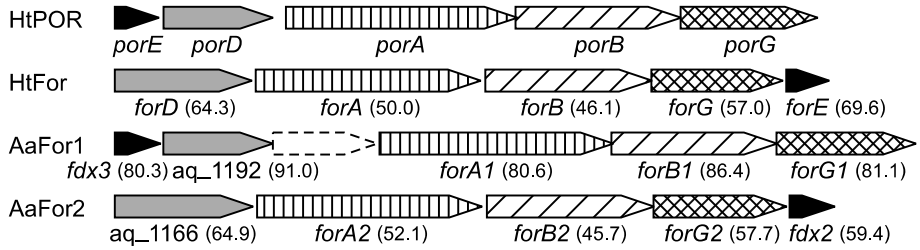

Fig. 2. Arrangement of the gene clusters for the OR enzymes from *H. thermophilus* and *A. aeolicus*. Homologous genes are shown in the same pattern. Numbers in parentheses indicate amino acid identity with the deduced sequences of the corresponding *por* genes. The dotted arrow box indicates aq_1194, which encodes a protein of unknown function (AAC07222). Ht, *H. thermophilus*; Aa, *A. aeolicus*.

in *E. coli* (Fig. 1B). These plasmids carried the fragments of *porEDABG*, *porDABG*, *porEABG*, and *porABG*, respectively. *E. coli* JM109 was transformed with the plasmids and cultivated under microaerobic conditions in the presence of 1 mM IPTG. Cell extracts of the recombinant *E. coli* cells were heat-treated to denature most of the host proteins, and the supernatants were used for the enzyme assay. POR activity was determined by the reduction of an artificial electron acceptor, methyl viologen, which was coupled to the oxidative decarboxylation of pyruvate under anaerobic conditions. Maximal activity was obtained when all five of the genes, *porEDABG*, were expressed from pUC-POR, supporting the five-subunit structure of the *H. thermophilus* POR (Table 1). In the absence of *porD* (pUC-PdD and pUC-PdED), no active enzyme was produced, indicating that *porD* was necessary for the expression of the holoenzyme. Similarly, cell extract of *E. coli* transformed with pUC-PdE showed only slight activity, suggesting that *porE* plays an important role in the POR reaction.

Purification of the recombinant POR

The *porEDABG* genes were expressed in *E. coli* JM109 harboring pUC-POR under the control of the *lac* promoter of the pUC vector. Since POR is sensitive to oxygen, purification of the recombinant POR was carried out under anoxic conditions. The recombinant POR was purified 2.9-fold with a recovery of 44% from the cell extract of the recombinant *E. coli* cells (Table 2). On SDS–PAGE, the purified recombinant POR showed an approximately 8-kDa protein band in addition to the previously identified four subunits (Fig. 3). The N-terminal amino acid sequence of this protein was identical to the deduced sequence of *porE* (data not shown), confirming the five-subunit structure of *H. thermophilus* POR. However, the intensity of the Coomassie brilliant blue (CBB)-stained PorE protein was significantly lower than those of the other subunits. The native molecular mass was determined to be 265 ± 10 kDa

Table 1
Specific activity of the crude POR in the heat-treated cell extract of the recombinant *E. coli* cells

Plasmid	Gene present	Specific activity (U mg^{-1})[a]
pUC19		0.01 ± 0.01
pUC-POR	*porEDABG*	4.17 ± 0.20
pUC-PdE	*porDABG*	0.11 ± 0.06
pUC-PdD	*porEABG*	0.01 ± 0.00
pUC-PdED	*porABG*	0.01 ± 0.01

[a] The values are means ± standard deviations from three independent experiments.

Table 2
Purification of the recombinant POR from *E. coli*[a]

Step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Recovery (%)	Purification (-fold)
Cell extract	270	155	1.74	100	1
Heat-treatment	248	61.1	4.05	92	2.3
Q Sepharose	130	29.8	4.56	48	2.6
Hydroxyapatite	118	24.6	4.98	44	2.9

[a] The starting material was 4.1 g wet weight of cells.

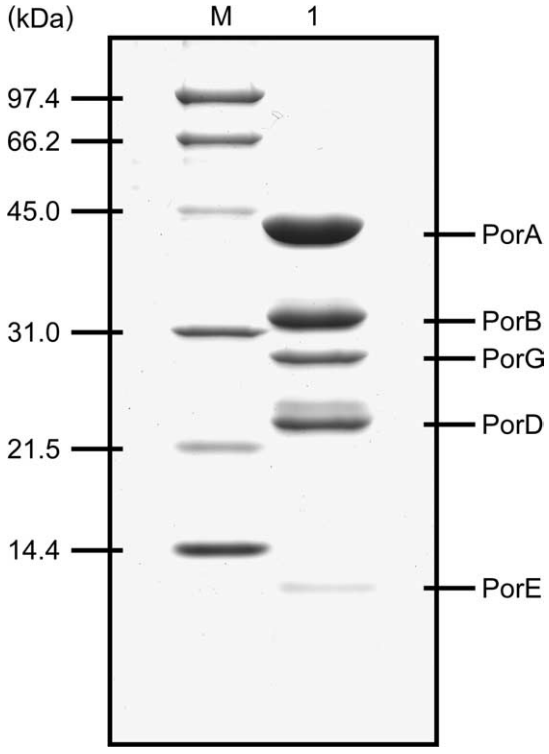

Fig. 3. SDS–PAGE (15%) of the recombinant POR. Lane 1, purified recombinant POR; lane M, molecular mass markers. Proteins were stained with CBB R-250.

by gel filtration chromatography, suggesting that the enzyme consists of two each of the five subunits. Enzymatic properties such as pH dependency and optimum temperature were essentially the same as those of the previously purified wild-type POR. The purified POR could reduce the two [4Fe–4S] ferredoxins of *H. thermophilus* [22] during pyruvate decarboxylation (data not shown).

Detection of PorE in the wild-type POR

To investigate whether the wild-type POR protein from *H. thermophilus* also contained PorE as a subunit, Western blot analysis was performed with the anti-PorE antibody prepared by using a 15-aa synthetic peptide, which corresponds to the N-terminus of PorE, as an antigen. Since PorE is highly similar to ForE (Fig. 2), the anti-PorE antibody also reacted with ForE (data not shown). To distinguish PorE from ForE on a Western blot, anaerobically grown *H. thermophilus* cells, in which the For enzyme is not expressed [19], were used. Cytoplasmic proteins present in the cells were fractionated at 50 kDa by ultrafiltration, separated on a non-denaturing gel, and activity-stained or transferred onto a PVDF membrane followed by immunostaining. The positive band on the Western blot was identical to the active POR band on the activity-stained gel (Fig. 4), indicating that PorE is a subunit in the POR holoenzyme. Broad bands below those of the POR holoenzyme were probably due to non-specific binding of the antibody (Fig. 4, lanes 4 and 6). No positive band was detected in the low molecular mass fraction (<50 kDa), indicating that PorE does not exist as a free protein.

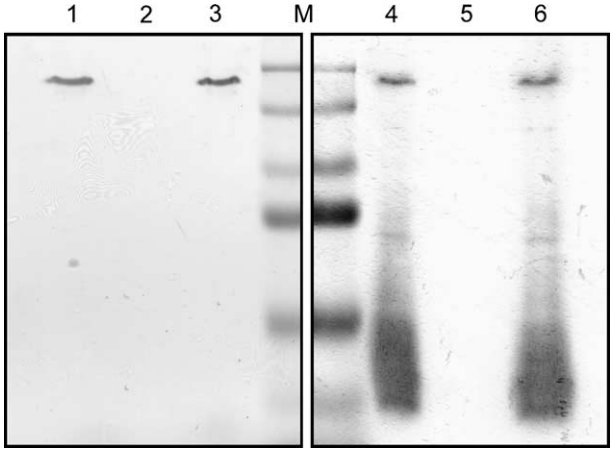

Fig. 4. Detection of the PorE protein in the cell extract of *H. thermophilus*. Cytoplasmic proteins were fractionated at 50 kDa by ultrafiltration with an Ultrafree-15 centrifugal filter device (Biomax-50 membrane; Millipore) and separated on a non-denaturing 10% polyacrylamide gel. Left panel: POR was activity-stained. Right panel: Proteins were transferred onto a PVDF membrane and immunostained using the anti-PorE antiserum. Lanes 1 and 4, cell extract; lanes 2 and 5, low molecular mass fraction (<50 kDa); lanes 3 and 6, high molecular mass fraction (>50 kDa). Lane M, pre-stained marker proteins.

Discussion

In this report, we cloned the genes encoding POR and investigated the quaternary structure of the enzyme. The results obtained by SDS–PAGE (Fig. 3) and immunological analysis (Fig. 4) clearly indicated that *H. thermophilus* POR consists of five distinct subunits as is the case of the For enzyme from this bacterium [18]. PorE was not detected in the previous work, most likely because it was very small (approximately 8 kDa) and stained poorly on SDS gels compared to the other four subunits (Fig. 3), as seen with other small acidic proteins [23]. PorE was similar to free ferredoxins as well as the δ subunit of the four subunit-type OR enzymes. However, PorE was not detected in the low molecular mass fraction (<50 kDa) on Western blots (Fig. 4), whereas Fd1 (7.9 kDa), a ferredoxin from this bacterium [22], was detected mainly in this fraction when a similar experiment was performed (data not shown). These results indicate that PorE exists as a subunit of POR, but not as a free ferredoxin.

PorE was predicted from its primary sequence to contain two ferredoxin-like iron–sulfur clusters, like the δ subunit of the four subunit-type ORs. These clusters were supposed to be involved in electron transfer between the other iron–sulfur cluster located in PorB, which is proximal to the active center, TPP, and external electron carriers [24]. POR lacking PorE expressed from pUC-PdE showed slight activity (Table 1). This is probably because electrons that were generated during the decarboxylation of pyruvate could not be transferred to an external electron acceptor via PorE but partially leaked from the iron–sulfur cluster of PorB to the artificial electron carrier, methyl viologen.

Although the native molecular mass of the wild-type POR had been reported to be 135 kDa [14], our results clearly indicate that the molecular mass of the recombinant POR was 265 ± 10 kDa, suggesting that the enzyme is a heterodecamer composed of two each of the five distinct subunits. The For enzyme of *H. thermophilus* was also predicted to be a heterodecamer [19]. Since most OR enzymes are composed of two catalytic units [7], the active enzyme of POR or For of *H. thermophilus* might be a homodimer of catalytic units that consists of five distinct subunits (PorABGDE or ForABGDE) and has a molecular mass of approximately 135 kDa. High concentrations of detergent and/or oxygen in the elution buffer might have caused the dissociation of the catalytic units of the wild-type POR during gel filtration chromatography in the previous work.

It has been proposed that ORs have evolved from an ancestral four subunit-type enzyme to the single- and two-subunit types [4,6,7]. Four of the five subunits of the *H. thermophilus* POR or For enzymes, PorABGE or ForABGE, are similar to the α, β, γ, and δ subunits of the four subunit-type enzymes, respectively. The specific motifs of each subunit are also conserved [18]. These results indicate that the POR and For enzymes of *H. thermophilus* are also the evolutionary relatives of the four subunit-type ORs. However, the additional subunits, PorD and ForD, are not similar to the other

OR family enzymes although they are essential for POR and For enzyme activity (Table 1; [18]). *Aquifex aeolicus* has two sets of gene clusters that encode putative OR enzymes (*fdx3*-aq_1192-[aq_1194]-*forA1B1G1* and aq_1166-*forA2B2G2*-*fdx2*) [25]. Their sequences and gene arrangements are highly similar to those of the *por* and *for* genes from *H. thermophilus* (Fig. 2), suggesting that they might also encode five subunit-type OR enzymes. As far as we know, proteins similar to PorD and ForD are encoded only in the genome of *H. thermophilus* and *A. aeolicus*. Both strains assimilate carbon dioxide via the RTCA cycle. Although the function of the novel subunits could not be predicted from their primary sequences, the subunits might be involved in the catalytic efficiency of anabolic carboxylation in these organisms.

Recently, POR has been purified from a methanogenic archaeon, *Methanococcus maripaludis*, and identified to consist of five distinct polypeptides [26], four of which correspond to subunits of the four subunit-type OR enzymes as *H. thermophilus* POR. The fifth polypeptide of *M. maripaludis* POR is a polyferredoxin-like protein, which is thought to be involved in electron transfer [27]. Since *H. thermophilus* POR and *M. maripaludis* POR are relatively distant from each other in the phylogenetic tree of the OR enzymes [28] and their novel fifth subunits have no similarity to each other, these ORs might have been evolved independently from an ancestral four subunit-type enzyme by the acquisition of the fifth polypeptides.

Acknowledgments

We thank Dr. Ki-Seok Yoon for help in anaerobic protein purification and for helpful discussions. This research was supported in part by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

References

[1] J.M. Blamey, M.W.W. Adams, Characterization of an ancestral type of pyruvate ferredoxin oxidoreductase from the hyperthermophilic bacterium, *Thermotoga maritima*, Biochemistry 33 (1994) 1000–1007.
[2] L. Pieulle, B. Guigliarelli, M. Asso, F. Dole, A. Bernadac, E.C. Hatchikian, Isolation and characterization of the pyruvate-ferredoxin oxidoreductase from the sulfate-reducing bacterium *Desulfovibrio africanus*, Biochim. Biophys. Acta 1250 (1995) 49–59.
[3] H.L. Drake, S.-I. Hu, H.G. Wood, Purification of five components from *Clostridium thermoaceticum* which catalyze synthesis of acetate from pyruvate and methyltetrahydrofolate. Properties of phosphotransacetylase, J. Biol. Chem. 256 (1981) 11137–11144.
[4] Q. Zhang, T. Iwasaki, T. Wakagi, T. Oshima, 2-Oxoacid:ferredoxin oxidoreductase from the thermoacidophilic archaeon, *Sulfolobus* sp. strain 7, J. Biochem. 120 (1996) 587–599.
[5] L. Kerscher, D. Oesterhelt, Purification and properties of two 2-oxoacid:ferredoxin oxidoreductases from *Halobacterium halobium*, Eur. J. Biochem. 116 (1981) 587–594.
[6] A. Kletzin, M.W.W. Adams, Molecular and phylogenetic characterization of pyruvate and 2-ketoisovalerate ferredoxin oxidoreductases from *Pyrococcus furiosus* and pyruvate ferredoxin oxidoreductase from *Thermotoga maritima*, J. Bacteriol. 178 (1996) 248–257.
[7] M.W.W. Adams, A. Kletzin, Oxidoreductase-type enzymes and redox proteins involved in fermentative metabolisms of hyperthermophilic archaea, Adv. Protein Chem. 48 (1996) 101–180.
[8] T. Kawasumi, Y. Igarashi, T. Kodama, Y. Minoda, Isolation of strictly thermophilic and obligately autotrophic hydrogen bacteria, Agric. Biol. Chem. 44 (1980) 1985–1986.
[9] T. Kawasumi, Y. Igarashi, T. Kodama, Y. Minoda, *Hydrogenobacter thermophilus* gen. nov., sp. nov., an extremely thermophilic, aerobic, hydrogen-oxidizing bacterium, Int. J. Syst. Bacteriol. 34 (1984) 5–10.
[10] S. Burggraf, G.J. Olsen, K.O. Stetter, C.R. Woese, A phylogenetic analysis of *Aquifex pyrophilus*, Syst. Appl. Microbiol. 15 (1992) 352–356.
[11] C. Pitulle, Y. Yang, M. Marchiani, E.R.B. Moore, J.L. Siefert, M. Aragno, P. Jurtshuk Jr., G.E. Fox, Phylogenetic position of the genus *Hydrogenobacter*, Int. J. Syst. Bacteriol. 44 (1994) 620–626.
[12] H. Shiba, T. Kawasumi, Y. Igarashi, T. Kodama, Y. Minoda, The CO_2 assimilation via the reductive tricarboxylic acid cycle in an obligately autotrophic, aerobic hydrogen-oxidizing bacterium, *Hydrogenobacter thermophilus*, Arch. Microbiol. 141 (1985) 198–203.
[13] M. Beh, G. Strauss, R. Huber, K.O. Stetter, G. Fuchs, Enzymes of the reductive citric acid cycle in the autotrophic eubacterium *Aquifex pyrophilus* and in the archaebacterium *Thermoproteus neutrophilus*, Arch. Microbiol. 160 (1993) 306–311.
[14] K.-S. Yoon, M. Ishii, T. Kodama, Y. Igarashi, Purification and characterization of pyruvate:ferredoxin oxidoreductase from *Hydrogenobacter thermophilus* TK-6, Arch. Microbiol. 167 (1997) 275–279.
[15] K.-S. Yoon, M. Ishii, T. Kodama, Y. Igarashi, Carboxylation reactions of pyruvate:ferredoxin oxidoreductase and 2-oxoglutarate:ferredoxin oxidoreductase from *Hydrogenobacter thermophilus* TK-6, Biosci. Biotechnol. Biochem. 61 (1997) 510–513.
[16] K.-S. Yoon, M. Ishii, Y. Igarashi, T. Kodama, Purification and characterization of 2-oxoglutarate:ferredoxin oxidoreductase from a thermophilic, obligately chemolithoautotrophic bacterium, *Hydrogenobacter thermophilus* TK-6, J. Bacteriol. 178 (1996) 3365–3368.
[17] N.-R. Yun, H. Arai, M. Ishii, Y. Igarashi, The genes for anabolic 2-oxoglutarate:ferredoxin oxidoreductase from *Hydrogenobacter thermophilus* TK-6, Biochem. Biophys. Res. Commun. 282 (2001) 589–594.
[18] N.-R. Yun, M. Yamamoto, H. Arai, M. Ishii, Y. Igarashi, A novel five-subunit-type 2-oxoglutarate:ferredoxin oxidoreductase from *Hydrogenobacter thermophilus* TK-6, Biochem. Biophys. Res. Commun. 292 (2002) 280–286.
[19] M. Yamamoto, H. Arai, M. Ishii, Y. Igarashi, Characterization of two different 2-oxoglutarate:ferredoxin oxidoreductases from *Hydrogenobacter thermophilus* TK-6, Biochem. Biophys. Res. Commun. 312 (2003) 1297–1302.
[20] J. Shine, L. Dalgarno, Determinant of cistron specificity in bacterial ribosomes, Nature 254 (1975) 34–38.
[21] E. Chabrière, M.-H. Charon, A. Volbeda, L. Pieulle, E.C. Hatchikian, J.-C. Fontecilla-Camps, Crystal structures of the key anaerobic enzyme pyruvate:ferredoxin oxidoreductase, free and in complex with pyruvate, Nat. Struct. Biol. 6 (1999) 182–190.
[22] T. Ikeda, M. Yamamoto, H. Arai, D. Ohmori, M. Ishii, Y. Igarashi, Two tandemly arranged ferredoxin genes in the *Hydrogenobacter thermophilus* genome: Comparative characterization of the recombinant [4Fe–4S] ferredoxins, Biosci. Biotechnol. Biochem. 69 (2005) 1172–1177.
[23] K.-S. Yoon, C. Bobst, C.F. Hemann, R. Hille, F.R. Tabita, Spectroscopic and functional properties of novel 2[4Fe–4S] cluster-containing ferredoxins from the green sulfur bacterium *Chlorobium tepidum*, J. Biol. Chem. 276 (2001) 44027–44036.
[24] S.W. Ragsdale, Pyruvate ferredoxin oxidoreductase and its radical intermediate, Chem. Rev. 103 (2003) 2333–2346.
[25] G. Deckert, P.V. Warren, T. Gaasterland, W.G. Young, A.L. Lenox, D.E. Graham, R. Overbeek, M.A. Snead, M. Keller, M. Aujay, R. Huber, R.A. Feldman, J.M. Short, G.J. Olsen, R.V. Swanson, The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*, Nature 392 (1998) 353–358.
[26] W.C. Lin, Y.-L. Yang, W.B. Whitman, The anabolic pyruvate oxidoreductase from *Methanococcus maripaludis*, Arch. Microbiol. 179 (2003) 444–456.

[27] W. Lin, W.B. Whitman, The importance of *porE* and *porF* in the anabolic pyruvate oxidoreductase of *Methanococcus maripaludis*, Arch. Microbiol. 181 (2004) 68–73.

[28] O. Schmitz, J. Gurke, H. Bothe, Molecular evidence for the aerobic expression of *nifJ*, encoding pyruvate:ferredoxin oxidoreductase, in cyanobacteria, FEMS Microbiol. Lett. 195 (2001) 97–102.