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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Characterization of a novel dye-linked L-proline dehydrogenase from an aerobic hyperthermophilic archaeon, *Pyrobaculum calidifontis*

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Abstract The activity of a dye-linked L-proline dehydrogenase (dye-L-proDH) was found in the crude extract of an aerobic hyperthermophilic archaeon, *Pyrobaculum calidifontis* JCM 11548, and was purified 163-fold through four sequential chromatography steps. The enzyme has a molecular mass of about 108 kDa and is a homodimer with a subunit molecular mass of about 46 kDa. The enzyme retained more than 90% of its activity after incubation at 100 °C for 120 min (pH 7.5) or after incubation at pHs 4.5–9.0 for 30 min at 50 °C. The enzyme catalyzed L-proline dehydrogenation to Δ^1-pyroline-5-carboxylate using 2,6-dichloroindophenol (DCIP) as the electron acceptor and the Michaelis constants for L-proline and DCIP were 1.67 and 0.026 mM, respectively. The prosthetic group on the enzyme was identified as flavin adenine dinucleotide by high-performance liquid chromatography. The subunit N-terminal amino acid sequence was MYDYVVV GAG. Using that sequence and previously reported genome information, the gene encoding the enzyme (Pcal_1655) was identified. The gene was then cloned and expressed in *Escherichia coli* and found to encode a polypeptide of 415 amino acids with a calculated molecular weight of 46,259. The dye-L-proDH gene cluster in *P. calidifontis* inherently differs from those in the other hyperthermophiles reported so far.

Keywords L-Proline dehydrogenase · FAD containing amino acid dehydrogenase · Hyperthermophilic archaeon · *Pyrobaculum calidifontis*

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Introduction

Dye-linked dehydrogenases (dye-DHs) belong to a group of oxidoreductases that catalyze the oxidation of various organic acids, amino acids, and alcohols in the presence of an artificial acceptor, like 2,6-dichloroindophenol (DCIP) or potassium ferricyanide (Ohshima and Tanaka 1993; Sakuraba et al. 2001). Many kinds of dye-DHs have been found in mesophilic microorganisms, and their use as specific elements in biosensors has been anticipated (Frew and Hill 1987). So far, however, their instability has limited the practical application of mesophilic dye-DHs. On the other hand, enzymes from hyperthermophiles are known to be much more stable than those from mesophiles and moderate thermophiles. This increases our ability to obtain useful information about their structure and function and makes them more amenable to practical application (Sakuraba et al. 2001; Kawakami et al. 2005; Satomura et al. 2008).

We have been extensively screening dye-DHs from hyperthermophiles for several years. During that period, we have identified two kinds of dye-linked L-proline dehydrogenase (dye-L-proDH) from several anaerobic hyperthermophiles, including *Thermococcus profundus*

(Sakuraba et al. 2001) and *Pyrococcus horikoshii* OT-3 (Kawakami et al. 2005), a dye-linked D-proline dehydrogenase from the anaerobe *Pyrobaculum islandicum* (Satomura et al. 2002) and a dye-linked D-lactate dehydrogenase from the aerobe *Sulfolobus tokodaii* (Satomura et al. 2008). We found that these enzymes remain stable for longer times under a variety of conditions and are applicable to a novel type of electrochemical detector for L-proline and D-proline (Kawakami et al. 2004; Tani et al. 2008). In addition, we just recently identified a dye-L-proDH from an aerobic hyperthermophilic archaeon *P. calidifontis*, isolated from a hot spring in the Philippines (Amo et al. 2002), and found that some enzymological properties of this enzyme are completely different from those of the *Thermococcus* and *Pyrococcus* strains. In this paper, we describe the purification and characterization of the *P. calidifontis* enzyme and the cloning of its gene and propose that this enzyme is a novel type of dye-L-proDH.

Materials and methods

Microorganism strains, plasmids, and chemicals

P. calidifontis JCM 11548 was obtained from the Japan Collection of Microorganisms (Wako, Japan). *Escherichia coli* strains JM109 and BL21(DE3) codon plus RIL were from Stratagene (La Jolla, CA, USA). The plasmid pET22b was from Novagen (Madison, WI, USA). DCIP and L-proline were purchased from Nacalai Tesque (Kyoto, Japan). Restriction endonucleases were from New England Biolabs (Beverly, MA, USA). All other chemicals were of reagent grade.

Conditions of cell growth

P. calidifontis was cultured in 2-L Erlenmeyer flasks in medium (600 mL, pH 7.0, adjusted with NaOH) containing 10 g L^{-1} of tryptone, 1 g L^{-1} yeast extract, and 3 g L^{-1} $Na_2S_2O_3 \cdot 5H_2O$ at 90 °C under aerobic conditions. After 24 h in culture, the cells (1.2 L medium) were harvested by centrifugation at 10,000×*g* for 10 min, washed first with 0.85% NaCl, and then with 10 mM potassium phosphate buffer (pH 7.2), and stored at −20 °C until use.

Enzyme assay and protein determination

Dye-L-proDH activity was assayed by measuring the rate of DCIP reduction at 50 °C. The standard reaction mixture contained 100 mM L-proline, 0.1 mM DCIP, 200 mM Tris–HCl buffer (pH 8.0) and the enzyme in a total volume of 1.0 mL. The mixture without the substrate (L-proline) was incubated at 50 °C for about 3 min in a cuvette with a 0.4-cm light path length, after which the reaction was started by the addition of L-proline. The initial decrease in the absorbance at 600 nm was measured using a Shimadzu UV-160A recording spectrophotometer (Kyoto, Japan). One unit was defined as the amount of the enzyme catalyzing the reduction of 1 μmol of DCIP/min at 50 °C. An absorption coefficient (ε) of 21.5 mM^{-1} cm^{-1} at 600 nm was used for DCIP (Ohshima and Tanaka 1993). The reductions in ferricyanide, *p*-iodonitrotetrazolium violet (INT), and horse liver cytochrome *c* were monitored at 405 nm (ε= 1.04 mM^{-1} cm^{-1}), 490 nm (ε=15.0 mM^{-1} cm^{-1}), and 553 nm (ε=15.3 mM^{-1} cm^{-1}), respectively. For reduction in INT, phenazine methosulfate (PMS) was used as an electron transfer intermediate. Protein concentration was determined by the method of Bradford, using bovine serum albumin as the standard (Bradford 1976).

Purification of L-Pro DH from *P. calidifontis* cells

All steps in the purification procedure were carried out at a room temperature, using 10 mM potassium phosphate buffer (pH 7.2) as the standard buffer. *P. calidifontis* cells (about 2 g wet weight) suspended in standard buffer were disrupted by ultrasonication. After centrifugation at 10,000×*g* for 20 min to remove any remaining intact cells and the cell debris, the supernatant was used as the crude extract. The enzyme solution was applied to a DEAE-Toyopearl 650M column (2.8×17.0 cm; Tosoh, Tokyo) previously equilibrated with the standard buffer. The enzyme was eluted with a linear gradient of 0–0.5 M NaCl in standard buffer, after which the active fractions were pooled and dialyzed against the standard buffer. Thereafter, preparative slab polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Ohshima and Ishida (1992), and the enzyme was extracted from the gel pieces using the standard buffer. The resultant enzyme solution was applied to a Superdex 200 pg column (2.6× 60 cm; GE Healthcare UK Ltd, UK) previously equilibrated with the standard buffer and then eluted with the same buffer. The active fractions were pooled, and the solution was used for experimentation.

Polyacrylamide gel electrophoresis and molecular mass determination

Native PAGE using 7.5% polyacrylamide gel was performed according to the method of Davis (1964). Activity staining was performed at 50 °C in a mixture containing 0.3 M Tris–HCl buffer (pH 8.0), 100 mM L-proline, 0.04 mM PMS, and 0.1 mM INT until a red band of sufficient intensity was visible. Protein was stained using 0.025% Coomassie brilliant blue G-250 in 50% methanol and 10% acetic acid.

Sodium dodecyl sulfate (SDS)-PAGE was carried out using 15% polyacrylamide gel containing 0.1% SDS

according to the method of Leammli (1970). Precision Plus protein standards (BIO-RAD, Tokyo, Japan) were used as the molecular mass standards. The protein sample was boiled for 5 min in 10 mM Tris–HCl buffer (pH 7.0) containing 1% SDS and 1% 2-mercaptoethanol. Protein bands were visualized by staining with 0.025% Coomassie brilliant blue R-250.

The molecular masses of the native enzymes were determined by gel filtration column chromatography using a Superdex 200 pg column (2.6×60 cm) with thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), and ribonuclease A (13.7 kDa) serving as molecular standards (GE Healthcare UK Ltd).

Extraction and determination of flavin

The flavin compound from the enzyme was extracted using 1% perchloric acid (PCA) as described elsewhere (Sakuraba et al. 2001; Kawakami et al. 2005). After centrifugation to remove the precipitate formed, the flavin compound in the supernatant was identified using high-pressure liquid chromatography with a TSKgel ODS-80Ts column (4.6×150 mm; Tosoh, Tokyo). A linear gradient between 10 mM potassium phosphate buffer (pH 6.0) and methanol (100% *v*/*v*) was used for the elution. The flow rate was 1.0 mL/min, and the total elution time was 45 min. FAD and FMN were monitored by determining the absorbance at 260 nm. FAD content was determined spectrophotometrically as previously described (Kawakami et al. 2004). After solutions of the enzyme was denatured by 1% (*v*/*v*) PCA and the precipitants were removed by centrifugation, the resultant supernatants were used for determination of FAD content. FAD concentration was estimated using an extinction coefficient of 11.3 mM^{-1} cm^{-1} at 450 nm.

Identification of the reaction product

The reaction product after oxidation of L-proline was identified using thin layer chromatography as previously described (Satomura et al. 2002). The postulated reaction products are Δ^1-pyrroline-5-carboxylate or Δ^1-pyrroline-2-carboxylate, which are converted into glutamate or 4-aminobutyrate, respectively, through oxidation by H_2O_2 (Meister 1954).

N-terminal amino acid analysis

The N-terminal amino acid sequence of the enzyme was determined using an Edman degradation protein sequencer. The phenylthiohydantatoin derivatives (Phh-Xaa) were separated and identified using a PPSQ-10 protein sequencer (Shimadzu).

Cloning of the dye-L-proDH gene

Sequencing of the genome of *P. calidifontis* was completed in 2007 (http://www.genome.jp/kegg-bin/show_organism?org=pcl). On the basis of the N-terminal amino acid sequence of the original dye-L-proDH, the open reading frame of an FAD-dependent oxidoreductase homologue (ORF ID, Pcal_1655) was identified in the *P. calidifontis* genome using BLAST (Altschul et al. 1997). To collect the genomic DNA containing Pcal_1655, *P. calidifontis* cells were harvested by centrifugation (10,000×*g*, 10 min) and washed first with 0.85% NaCl and then with 10 mM potassium phosphate buffer (pH 7.2), after which the genomic DNA was prepared as described previously (Murray and Thompson 1980).

Overexpression and purification of the recombinant protein

To construct an expression plasmid, a 1.3-kbp gene fragment comprised of the gene encoding dye-L-proDH and *Nde*I and *Eco*RI linkers was amplified by PCR using the primers 5′-TGTCTCCATATGTATGACTATG-3′, which was designed to contain the N-terminal region of the dye-L-proDH gene and an *Nde*I digestion sequence, and 5′-GCCGTAGGAATTCCT CAATCATATCACAA-3′, which was designed to contain the C-terminal region and an *Eco*RI digestion sequence. The respective restriction sequences are underlined. The genomic DNA was used as the template. The amplified 1.3-kbp fragment was digested with *Nde*I and *Eco*RI and then ligated into the expression vector pET22b, linearized using *Nde*I and *Eco*RI, to generate pLPDH. Thereafter, *E. coli* strain BL21(DE3) codon plus RIL cells were transformed with pLPDH, and the transformants were cultivated at 37 °C in 1 L of Luria–Bertani medium containing 50 μg mL^{-1} ampicillin until the optical density at 600 nm reached 0.6. Expression was then induced by adding 0.1 mM isopropyl-β-D-thiogalacto-pyranoside to the medium, and cultivation was continued for an additional 4 h. The cells were then harvested by centrifugation, suspended in 10 mM potassium phosphate buffer (pH 7.2), which was used as the standard buffer, and disrupted by ultrasonication. The crude extract was heated at 70 °C for 30 min, and the denatured protein was removed by centrifugation (10,000×*g* for 10 min). The supernatant was purified by preparative slab PAGE, and the enzyme was extracted from the gel pieces using the standard buffer. The resultant enzyme solution was applied on a Superdex 200 pg column (2.6×60 cm) previously equilibrated with the standard buffer and eluted with the same buffer. The active fractions were pooled, and the solution was used for experimentation.

Kinetic parameters

The Michaelis constant (K_m) was determined from double-reciprocal plots of the initial rate data using L-proline or

L-hydoxyproline as the electron donor and DCIP as the electron acceptor at 50 °C.

Results

Purification of dye-L-proDH from *P. calidifontis*

Many dye-DHs are membrane-bound enzymes. With that in mind, we initially disrupted *P. calidifontis* cells by sonication and fractionated them into particulate and supernatant fractions by ultracentrifugation (Beckman Ultracentrifuge at 140,000×*g* for 90 min). Dye-L-proDH activity was then measured separately in the two fractions. More than 90% of the activity was found in the supernatant fraction, which indicates that the enzyme is either not bound to the cytoplasmic membrane or is easily solubilized from the membrane. We therefore omitted the step for solubilization and fractionation of the enzyme from the purification procedure. The purification of dye-L-proDH from *P. calidifontis* is summarized in Table 1. After the final step, the enzyme had been purified about 163-fold, with an overall yield of about 18%.

Molecular mass and subunit structure

Using native-gradient PAGE, the molecular mass of the *P. calidifontis* dye-L-proDH was determined to be about 108 kDa (data not shown); moreover, SDS-PAGE showed one major band (Fig. 1), indicating the enzyme is composed of identical subunits. The molecular mass of the subunit was estimated to be about 46 kDa, indicating that the enzyme is a homodimer.

pH and temperature optima and thermostability

To determine the pH optimum for this dye-L-proDH, its activity was measured at various pHs at 50 °C. Acetate (pH 4.5–5.5), MES-NaOH (pH 5.5–6.5), potassium phosphate (pH 6.0–7.5), MOPS-NaOH (pH 6.5–7.5), and Tris-HCl (pH 7.5–9.0) buffers were used, and the pH of the reaction mixture was measured at 25 °C after each assay. The optimum pH was about 7.0, and half of the activity was retained at pH 6.0 and 9.0.

We determined the optimum temperature for the enzyme by assaying activity at temperatures ranging from 50 to 90 °C. Because DCIP is unstable above about 70 °C, we used ferricyanide in 200 mM Tris-HCl buffer (pH 7.5) as the electron acceptor instead of DCIP. The assay was started by adding the enzyme after preincubation of the reaction mixture for 5 min at the temperature of interest. We found that the optimum temperature for dye-L-proDH activity was above 80 °C, but more precise determination was not possible due to heat-induced destruction of the reaction system.

When we assessed the thermostability of this dye-L-proDH, we found that it retained about 90% of its activity after incubation at 100 °C for 120 min, and the half-life at 105 °C was about 90 min (Fig. 2). In addition, when the enzyme was incubated at pHs between 4.5 and 9.0 for 30 min at 50 °C, it retained more than 90% of the highest activity seen at pH 7. Thus, dye-L-proDH from *P. calidifontis* appears to be very stable under a variety of conditions.

Substrate and electron acceptor specificity

We found that this dye-L-proDH catalyzed the dehydrogenation of L-proline as the most preferred substrate, though it also showed activity with L-hydroxyproline (relative activity, 72% as compared to L-proline). On the other hand, sarcosine, D-proline, L-ornithine, L-glutamate, L-arginine, L-serine, L-leucine, L-valine, and L-alanine were all inert as substrates. When we examined the electron acceptor specificity of the enzyme, we found that DCIP was the most preferred electron acceptor, though ferricyanide and PMS-INT also exhibited electron acceptor activity (relative activities, 93% and 9%, respectively, as compared to DCIP). NAD, NADP, and bovine heart cytochrome *c* were inert as electron acceptors.

Steady-state kinetics

We next carried out steady-state kinetic analysis of L-proline dehydrogenation with DCIP as the electron acceptor. Initial velocity experiments were done by varying the concentration of one substrate while keeping the concentration of the other substrates constant, as previously described (Ohshima and Tanaka 1993). Double reciprocal plots of the initial velocity and the substrate concentrations (L-proline and DCIP) showed a series of parallel lines. The

Table 1 Purification of dye-L-proDH from *P. calidifontis*

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	146	3.72	0.026	100	1
DEAE-Toyopearl	6.82	2.53	0.371	68	14
Preparative slab PAGE	0.67	1.05	1.567	28	60
Superdex 200 pg	0.16	0.68	4.250	18	163

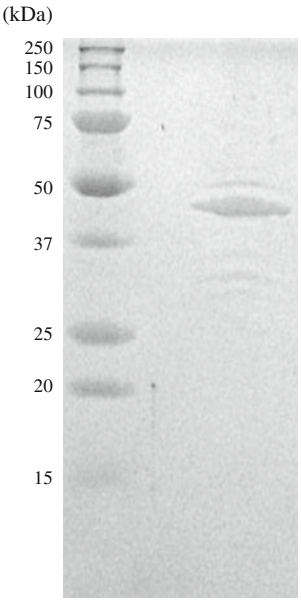

Fig. 1 SDS-PAGE of the purified enzyme. *Left column* molecular maker proteins, *right column* the enzyme

K_m values were calculated from the secondary plot of the intercepts vs. the reciprocal of the substrate concentration. From the secondary plots of the intercepts vs. reciprocals of L-proline and DCIP concentrations, the K_m value for L-proline and DCIP was determined to be 1.67 and 0.026 mM, respectively. The K_m value for L-hydroxyproline and DCIP was similarly calculated to be 6.05 and 0.035 mM, respectively. In addition, the K_{cat}/K_m value for L-proline (DCIP) and L-hydroxyproline (DCIP) were 1.25 (79.5) and 0.125 (21.9) s^{-1} mM^{-1}, respectively.

N-terminal amino acid sequences

Using an automated Edman degradation protein sequencer, the sequence of the ten N-terminal amino acid residues of the subunit was determined to be MYDYVVVGAG.

Amino acid sequence alignment of *P. calidifontis* dye-L-proDH

The similarity between the amino acid sequence of *P. calidifontis* dye-L-proDH and other enzymes was searched using the BLAST server in the Kyoto Encyclopedia of Genes and Genomes. We found that *P. calidifontis* dye-L-proDH exhibited a small degree of similarity to the *T. profundus* dye-proDH β subunit (PdhB, 16%; originally assigned as a putative sarcosine oxidase β subunit), and to the subunits of two different *Pc. horikoshii* ProDHs, PH1364 and PH1751 (26% and 25% identity, respectively; Fig. 3).

Expression dye-L-proDH gene in *E. coli* and purification of its product

E. coli BL21(DE3) codon plus RIL cells transformed with the expression vector pLPDH exhibited dye-L-proDH activity (total protein=251 mg, specific activity=0.048 U/mg) that was not lost after incubation at 70 °C for 30 min. We were then able to easily obtain the purified enzyme (1.8 mg) in three steps: heating of the cell extract at 70 °C for 30 min followed by successive preparative slab PAGE and Superdex 200 pg chromatography. The yield was about 19%.

Properties of recombinant dye-L-proDH

The pH and temperature optima, thermostability, and substrate specificity of the recombinant enzyme were nearly identical to those of the native enzyme (data not shown).

The spectrum of the recombinant dye-L-proDH showed the characteristics of typical flavoprotein (Fig. 4). The flavin prosthetic group was extracted from the enzyme using 1% PCA, and analysis by high-performance liquid chromatography revealed it to be FAD; no FMN was detected. The content of FAD was determined to be 0.74 mol of FAD per mole of enzyme. In addition, when the enzyme reaction product after oxidation by H_2O_2 was analyzed by thin layer chromatography, the enzyme reaction produced a ninhydrin spot corresponding to L-glutamate, but not 4-aminobutyrate (data not shown). This means that the reaction product was Δ^1-pyrroline-5-carboxylate (P5C), not Δ^1-pyrroline-2-carboxylate.

Discussion

In this study, we identified dye-L-proDH activity in an aerobic hyperthermophilic archaeon, *P. calidifontis*, and were able to purify and characterize the enzyme. Following cell disruption by sonication, the enzyme appeared in the soluble fraction without the need for a special solubilization procedure, suggesting that perhaps the enzyme is distributed on the surface of the cytoplasmic membrane and is easily released, which has been seen previously with dye-L-proline dehydro-

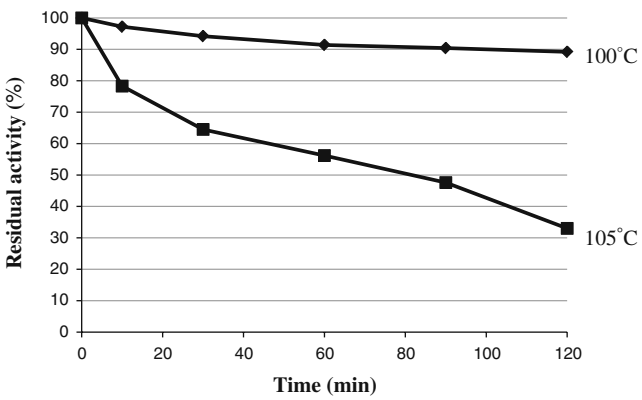

Fig. 2 The effect of temperature on dye-L-proDH. The enzyme was incubated at 100 °C (*filled diamond*) and 105 °C (*filled square*), and the residual activity was measured at 50 °C

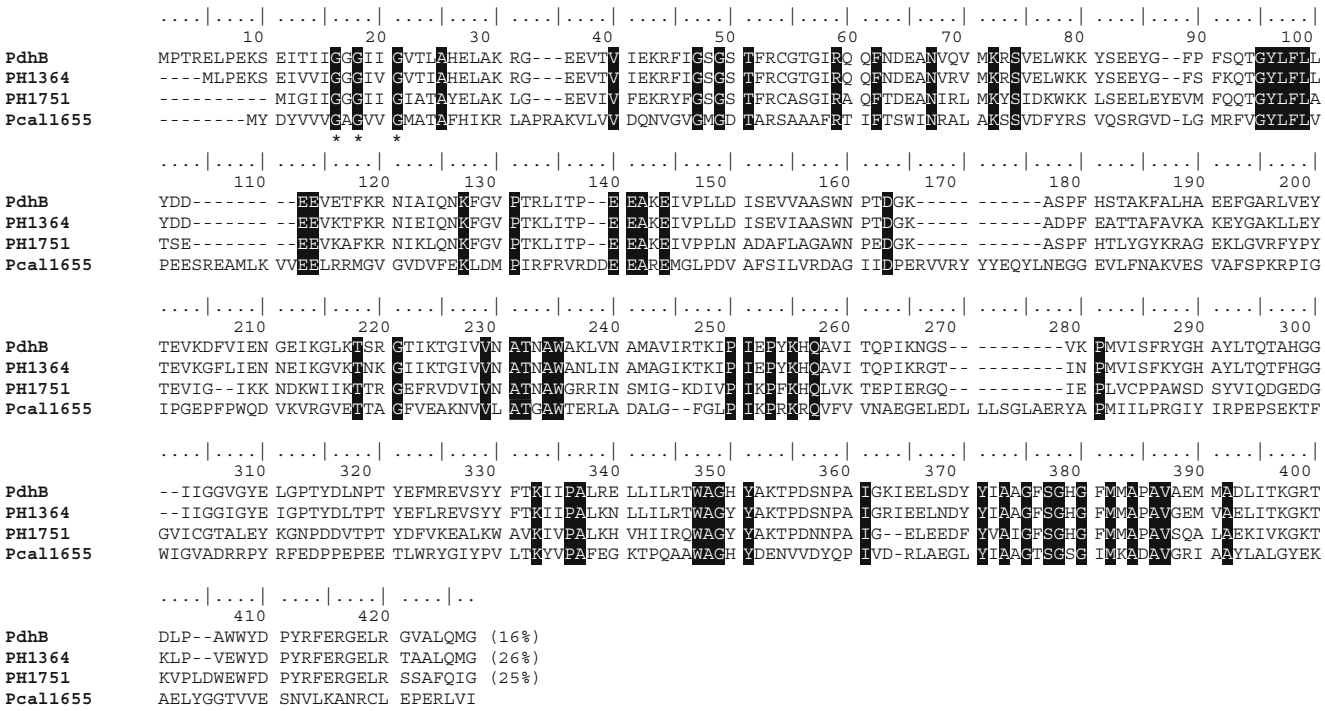

Fig. 3 Alignment of amino acid sequence of dye-L-proDH from four strains of hyperhtermophilic arachaea. Highly conserved residues are depicted in *black boxes*. Amino acid sequence identity with Pcal1655 is given in *parentheses*. The *asterisks* are the FAD-binding motif. Proteins shown on figure: *PdhB* dye-L-proDH β-subunit from *T. profundus* (NCBI accession number BAD13509.1); *PH1364* PDH1 β-subunit from *Pc. horikoshii* (NCBI accession number BAA30470.1); *PH1751* PDH2 β-subunit from *Pc.horikoshii* (NCBI accession number BAA30865.1); *Pcal1655* dye-L-proDH from *P. calidofontis* (NCBI accession number YP_001056538.1)

genases from *T. profundus* and *Pc. horikoshii* (Sakuraba et al. 2001, Kawakami et al. 2005) but not with a dye-D-proline dehydrogenase from *P. islandicum* (Satomura et al. 2002).

Two different types of dye-L-proDH, PDH1 and PDH2, have been identified in the anaerobic hyperthermophile *P. horikoshii* OT-3, belonging to the phylum Euryarchaeota (Kawakami et al. 2005). PDH1 is a heterooctameric complex ($\alpha 4\beta 4$; molecular mass, 440 kDa) containing FAD, FMN, Fe, and ATP (Kawakami et al. 2005), while PDH2 is a tetrameric complex ($\alpha\beta\gamma\delta$; molecular mass, 120 kDa). Structural analysis of the PDH1 complex showed the enzyme to be a unique diflavin dehydrogenase containing a novel electron transfer system that is totally different from that of the PDH2 complex, which contains four components: proDH, NADH dehydrogenase, a ferredoxin-like protein, and a functionally unknown protein (Tuge et al. 2005). The structure of β-subunit, which is a PDH1 catalytic component containing FAD as a cofactor, was similar to that of monomeric sarcosine oxidase (MSOX). Based on the structural comparison with MSOX, the residues forming a substrate-binding pocket could be predicted (Trickey et al. 1999). As shown in Fig. 3, several sequence motifs in β-subunit of the *Pc. horikoshii* PDH1 (PH1364) are well conserved in all dye-L-pro DHs. Among these motifs, ^{12}GXGXXG^{17} (residue numbers of PH1364 are given) is typical of FAD-binding domain. The residues in the motifs of ^{86}GYLF^{89}, ^{304}WAGXY^{308}, and ^{332}GXSGXGXM^{339} are involved in the construction of the binding sites for the substrate and isoalloxazine ring of FAD in β-subunit of the *Pc. horikoshii* PDH1. These motifs are conserved in all the dye-L-pro DHs. Thus, these motifs might be important for the catalytic role of dye-L-pro DHs.

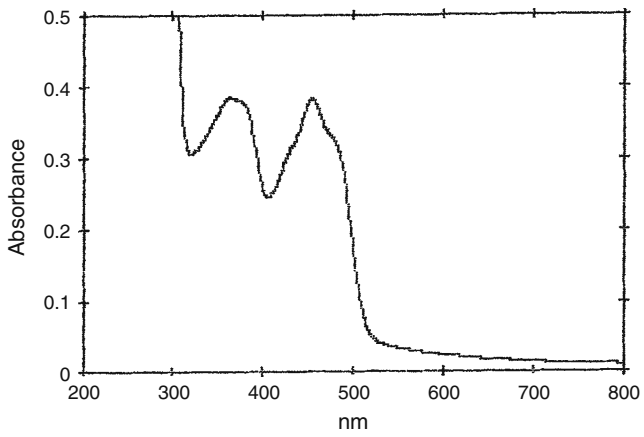

Fig. 4 Absorption spectra of recombinant dye-L-pro DH in 10 mM potassium phosphate (pH 7.2)

Here, we showed that, like PDH1 and PDH2, a dye-L-proDH from the aerobic hyperthermophilic archaeon *P. calidifontis*, belonging to the phylum Crenarchaeota, catalyzes the conversion of L-proline to P5C in the presence of DCIP, but it differs

from those other enzymes in that it has a homodimeric structure (2×46 kDa). Thus, the molecular structure of the *P. calidifontis* enzyme is totally unlike those of PDH1 and PDH2 (Kawakami et al. 2005). In addition, we found that the sequence of the *P. calidifontis* subunit shows little similarity to those of the PDH1 and PDH2 β-subunits (26% and 25% identity, respectively), which are responsible for L-proline dehydrogenation—i.e., *P. calidifontis* dye-L-proDH is inherently different from the two dye-L-proDHs from *Pc. horikoshii* OT-3, with respect to sequence. We have thus identified a novel type of dye-L-proDH expressed by the aerobic hyperethermophilic archaeon *P. calidifontis*.

In addition to L-proline, dye-L-proDH from *P. calidifontis* catalyzes the dehydrogenation of L-hydroxyproline. By contrast, neither PDH1 nor PDH2 are able to use L-hydroxyproline as a substrate. Thus, the substrate specificity of the *P. calidifontis* enzyme appears to be somewhat broader than those of the *Pc. horikoshii* and *T. profundus* enzymes. In addition, the *P. calidifontis* enzyme is still active after incubation at 100 °C for 120 min, which makes it more thermostable than the enzymes from *Pc. horikoshii* and *T. profundus*. We have developed an application for *T. profundus* enzymes in an electrochemical sensor used to assay L-proline (Zheng et al. 2006). In addition, the dye-D-proDH from *P. islandicum* has proven useful in a novel biosensor for determining levels D-amino acids, such as D-proline and D-valine, in biological samples. In that sensor, the electrode is prepared by immobilizing the thermostable dye-D-ProDH within an agar gel membrane. At temperatures above 80 °C, liquid agar and the enzyme solution can be mixed and simply co-immobilized on a glassy carbon electrode using the spin-coating method (Tani et al. 2008). Moreover, carbon nanotube gel, which is composed of a mixture of single-wall carbon nanotubes, an ionic liquid, and an immobilized thermostable dye-D-proDH, can serve as the basis for electrodes that enable determination of D-amino acid levels in food samples with greater sensitivity than is obtained with the glassy agar-based electrode described above (Tani et al. 2009). In these cases, the high thermostability of dye-D-proDH supports the development of novel types of electrodes. That said, *P. calidifontis* dye-L-proDH loses no activity with incubation at 100 °C for 120 min and is much more thermostable than the L-proline sensor enzyme, *T. profundus* L-proDH, which loses activity at temperatures above 80 °C. In addition, the simpler subunit structure (homodimer) of the *P. calidifontis* enzyme may facilitate its immobilization during preparation of enzyme electrodes, as compared to the *Pc. horikoshii* heterotetrameric and heterooctermeric enzymes.

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