The activity of dye-linked D-proline dehydrogenase was found in the crude extract of a hyperthermophilic archaeon, *Pyrobaculum islandicum* JCM 9189. The dye-linked D-proline dehydrogenase was a membrane associated enzyme and was solubilized from the membrane fractions by treatment with Tween 20. The solubilized enzyme was purified 34-fold in the presence of 0.1% Tween 20 by four sequential chromatographies. The enzyme has a molecular mass of about 145 kDa and consisted of homotetrameric subunits with a molecular mass of about 42 kDa. The N-terminal amino acid sequence of the subunit was MKVAVGGHGLFTAYHL-RQQGADVVI. The enzyme retained its full activity both after incubation at 80 °C for 10 min and after incubation in the range of pH 4.0–10.0 at 50 °C for 10 min. The enzyme-catalyzed dehydrogenation of several D-amino acids was carried out using 2,6-dichloroindophenol as an electron acceptor, and D-proline was the most preferred substrate among the D-amino acids. The Michaelis constants for D-proline and 2,6-dichloroindophenol were determined to be 4.2 and 0.14 mM, respectively. Δ1-Pyrroline-2-carboxylate was identified as the reaction product from D-proline by thin layer chromatography. The prosthetic group of the enzyme was identified to be FAD by high-performance liquid chromatography. The gene encoding the enzyme was cloned and expressed in *Escherichia coli*. The nucleotide sequence of the dye-linked D-proline dehydrogenase gene was determined and encoded a peptide of 363 amino acids with a calculated molecular weight of 40,341. The amino acid sequence of the *Pb. islandicum* enzyme showed the highest similarity (38%) with that of the probable oxidoreductase in *Sulfolobus solfataricus*, but low similarity with those of D-alanine dehydrogenases from the mesophiles so far reported. This shows that the membrane-bound D-proline dehydrogenase from *Pb. islandicum* is a novel FAD-dependent amino acid dehydrogenase.

Pyrobaculum islandicum is a continental hyperthermophilic archaeon that was isolated from boiling neutral sulfataric water from Iceland (1). Recently, the D-amino acid content in *Pb. islandicum* has been determined, and the occurrence of several D-amino acids, including L-serine, L-aspartate, and L-proline, was found at high levels (2). However, the physiological function and the metabolic pathway of the D-amino acids are still unknown, particularly in hyperthermophilic archaea. Therefore, the crude extract of *Pb. islandicum* cells was assayed for enzymatic activity using several D-amino acids. As a result, we found the activity of a dye-linked D-proline dehydrogenase, which catalyzed the dehydrogenation of D-proline in the presence of 2,6-dichloroindophenol (Cl2Ind). The activity was exclusively detected in the membrane fraction of *Pb. islandicum* after fractionation by ultracentrifugation. The presence of dye-linked dehydrogenases that catalyze the dehydrogenation of D-amino acids has been so far reported in *Pseudomonas aeruginosa* (3), *Pseudomonas fluorescens* (4), *Salmonella typhi murium* (5), *Escherichia coli* B (6), and *E. coli* K12 cells (7). However, these enzymes predominantly utilize L-alanine as the most preferable substrate. The dehydrogenase utilizing D-proline as the best electron donor has not been reported so far. In this paper, we report the solubilization, purification, characterization, and nucleotide sequencing of the dye-linked D-proline dehydrogenase from a hyperthermophilic archaeon, *Pb. islandicum* JCM 9189, and that the enzyme is a novel amino acid dehydrogenase.

EXPERIMENTAL PROCEDURES

Materials—Cl2Ind and D-proline were purchased from Wako Pure Chemical Industries. *alto*-4-Hydroxy-D-proline, FAD, FMN, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma and Tween 20 from Nacalai Tesque. All other chemicals were of reagent grade.

Microorganism and Growth Conditions—The hyperthermophilic archaeon *Pb. islandicum* JCM 9189 was obtained from the Japan Collection of Microorganisms. *Pb. islandicum* was cultured as described previously (8). The cells were harvested by centrifugation (10,000 x g for 10 min) and washed twice with 0.85% NaCl solution. The washed cells were suspended in 10 mM Tris/HCl buffer (pH 8.0) and stored at −20 °C.

Enzyme Assay and Protein Determination—Enzyme activity was assayed spectrophotometrically with a Shimadzu 160A spectrophotometer equipped with a thermostat. The standard reaction mixture was composed of 20 mM D-proline, 0.2 mM Cl2Ind, 200 mM Tris/HCl buffer (pH 8.0), and enzyme in a total volume of 0.5 ml. The mixture was incubated at 50 °C in a cuvette with a 0.2-cm light path. The reaction was started by the addition of Cl2Ind and was followed by measuring the initial decrease in absorbance at 600 nm. One enzyme unit was defined as the amount catalyzing the reduction of 1 μmol of Cl2Ind for 1 min. The millimolar absorption coefficient (ε max) of 19.1 mM−1 cm−1 at 600 nm was used for Cl2Ind (9). Reduction of ferricyanide, p-iodo-
nitroetrazolium violet (INT), and horse heart cytochrome c, methylene blue, and benzyl viologen were assayed at 420 nm (εmax = 9.7 mm−1 cm−1), 490 nm (εmax = 15.0), 550 nm (εmax = 13.5), 670 nm (εmax = 7.8), and 580 nm (εmax = 7.8), respectively (10). Protein concentration was determined by a BCA Protein assay reagent kit supplied by Pierce with bovine serum albumin as standard.

Cell Destruction and Fractionation—The cell suspension was passed twice through a French pressure cell at 6.9 megapascals. After removal of the unbroken cells by centrifugation at 10,000 × g for 10 min, the supernatant solution was further centrifuged at 140,000 × g for 1 h (Beckman Ultracentrifuge). The precipitate was used as the membrane fraction.

Solubilization of Dye-linked d-Proline Dehydrogenase from Pb. islandicum—Dye-linked d-proline dehydrogenase was solubilized by resuspending the membrane fraction in 10 mM Tris/His buffer (pH 8.0) containing 1% (w/v) Tween 20. The buffer (1 ml) was mixed with about 10 mg of the membrane protein. After stirring at room temperature (about 25°C) for 12 h, the insoluble materials were removed by centrifugation at 140,000 × g for 1 h.

Purification of Dye-linked d-Proline Dehydrogenase from Pb. islandicum—The entire operation was performed at room temperature (about 25°C). Glycerol (10% v/v), EDTA (0.1 mM), and Tween 20 (0.1% w/v) were added to all buffers used in purification steps. The solubilized dye-linked d-proline dehydrogenase was dialyzed against 20 mM potassium phosphate buffer (pH 6.0) containing 1% (w/v) sodium deoxycholate, 0.1% (w/v) 2-mercaptoethanol, and glycerol (10% v/v), EDTA (0.1 mM), and Tween 20 (0.1% w/v) as described elsewhere (9, 13). After the removal of the precipitate formed by centrifugation, the supernatant was used to identify the flavin compound by high-performance liquid chromatography (HPLC) with a TSK gel ODS-80 Ts column (4.6 × 150 mm, Tosoh). A linear gradient between 10 mM potassium phosphate buffer containing methanol (20% v/v) and methanol was used for the elution. The flow rate was 1.0 ml/min, and the total elution time was 15 min. FAD and FMN were monitored by the absorbance at 260 nm.

N-terminal Amino Acid Sequence—The N-terminal amino acid sequence of the enzyme was analyzed with an automated Edman degradation protein sequencer. The phenylthiohydantoin-derivatives (PTH-Xaa) were separated and identified using the protein sequencer PPSQ-10 (Shimadzu).

Construction of Genomic Library and Screening of the Positive Clone—The genomic DNA from Pb. islandicum was prepared by Genomic Prep™ Cell and Tissue DNA Isolation kit (Amersham Biosciences). The DNA yield was 2–2.2 mg of 2 g of cells. For screening the dye-linked d-proline dehydrogenase gene, the genomic DNA preparation was digested with XbaI and/or PvuII. Bacillus subtilis pUC18 was also digested with XbaI and treated with alkaline phosphatase (New England Biolabs). It was ligated with the genomic DNA XbaI fragments from Pb. islandicum and used to transform E. coli JM 109 as a library for screening of the dye-linked d-proline dehydrogenase gene. Colony hybridization was done as follows. The recombinant colonies from the genomic library were transferred and fixed onto the nylon membranes (Hybond-N; Amersham Biosciences); the membranes were then incubated with 3× SSC, 0.05% sodium pyrophosphate, and 100 µg/ml salmon testes DNA (Sigma). The membranes were then hybridized in a mixture containing 6 × SSC, 5 × Denhardt’s solution, 0.05% SDS, 0.05% sodium pyrophosphate, and 100 µg/ml salmon testes DNA (Sigma), and 500,000 cpm/ml of 32P-labeled probe at 53 °C for 48 h. The membranes were then washed twice with washing buffer (6 × SSC and 0.05% sodium pyrophosphate) at 53 °C for 20 min. The positive clones were detected with BAS-1500 system (Fuji film).

DNA Sequencing and Analysis—The nucleotide sequence was determined by the dideoxy-chain termination method (14) using an automated DNA Sequencer 377A (Applied Biosystems). The nucleotide sequence was analyzed using GENETYX gene analysis software (Software Developments Co., Ltd.). The nucleotide sequence reported in this paper has been submitted to the DDBJ, GenBank™, and EBI data banks under accession number AB071692.

Expression of the Dye-linked d-Proline Dehydrogenase Gene—For construction of the expression plasmid, a 1.0-kbp gene fragment, which consisted of the structural gene of Pb. islandicum dye-linked d-proline dehydrogenase, and NdeI and BamHI linkers were amplified by PCR with the following two primers. The first was designed to contain the N-terminal region of the dye-linked d-proline dehydrogenase gene and the NdeI digestion sequence (5′-ATCTGCATATGAAAGGTTCGTTGAGGT-3′) and the second was to contain the C-terminal region and the BamHI digestion sequence (5′-TAGGATCCCTAGGGCGCGGAAAAGGCGG-3′). The pDPDH2 plasmid DNA was used as the template. The amplified 1.0-kbp fragment was digested with NdeI and BamHI and ligated with the expression vector pET-11a (Novagen) linearized with NdeI and BamHI to generate pDPDH3. E. coli strain
Dye-linked d-Proline Dehydrogenase from a Hyperthermophilic Archaeon

TABLE I

| Step                      | Total protein (mg) | Total activity units | Specific activity units/mg | Yield (%) |
|---------------------------|-------------------|----------------------|-----------------------------|-----------|
| Crude extract             | 1204              | 190                  | 0.157                       | 100       |
| Membrane fraction         | 451               | 229                  | 0.507                       | 121       |
| Solubilization            | 131               | 201                  | 1.53                        | 104       |
| Red Sepharose CL-4B       | 51.3              | 124                  | 2.42                        | 65        |
| GIGAPITE                  | 14.3              | 101                  | 0.76                        | 53        |
| Uno Q                     | 7.40              | 82.6                 | 11.2                        | 43        |
| Superdex 200 prep grade   | 3.70              | 19.6                 | 5.30                        | 10        |

BL21-CodonPlus™ cells (Stratagene) were transformed by the recombinant plasmids and plated on Luria-Bertani medium with ampicillin (50 μg/mL).

Purification of the Dye-linked d-Proline Dehydrogenase from Recombinant E. coli Cells—The transformants were grown to OD_{600} of 0.5 at 37 °C in a medium containing 12 g of tryptone, 24 g of yeast extract, 5 ml of glycerol, 12.5 g of K_{2}HPO_{4}, 3.8 g of KH_{2}PO_{4}, and 50 mg of ampicillin. After induction with 1 mM isopropyl-β-D-thiogalactopyranoside for 4 h incubation, the cells were harvested and washed twice with 0.85% NaCl solution. The washed cells were suspended in 10 mM Tris/HCl buffer (pH 8.0). The cell debris was removed by centrifugation (10,000 x g for 10 min), and the supernatant solution was used as the crude extract. The enzyme solution was incubated at 80 °C for 10 min. Denatured proteins were separated by centrifugation (10,000 x g for 10 min). The resulting supernatant was used for further purification.

The entire operation was performed at room temperature (about 25 °C). Glycerol (10% v/v) and Tween 20 (0.1% w/v) were added to all buffers used in purification steps. The recombinant dye-linked d-proline dehydrogenase was dialyzed against 20 mM Tris/HCl buffer (pH 9.0). The enzyme solution was placed on a DEAE-Toyopearl 650 M column (TOSOH, 2.0 x 10 cm) equilibrated with 20 mM Tris/HCl buffer (pH 9.0). After the column was washed with the same buffer, the enzyme was eluted with a linear gradient of NaCl concentration (0 to 0.5 M). The active fractions were pooled and dialyzed against 20 mM Tris/HCl buffer (pH 9.0). The enzyme solution was placed on a Q-Sepharose column (Amersham Biosciences, 1.3 x 10 cm) equilibrated with 20 mM Tris/HCl buffer (pH 9.0). After the column was washed with the same buffer, the enzyme was eluted with a linear gradient of NaCl concentration (0 to 0.3 M). The active fractions were pooled and dialyzed against 10 mM Tris/HCl buffer (pH 8.0).

RESULTS

Distribution and Purification of Dye-linked d-Proline Dehydrogenase—Many dye-linked dehydrogenases are known to be membrane-bound enzymes. We tested whether the dye-linked d-proline dehydrogenase was present in a soluble or membrane-binding form. The cells of *Pc. islandicum* were broken using a French press cell, and the extract was fractionated into the particulated and supernatant fractions by ultracentrifugation. The activity of dye-linked d-proline dehydrogenase in the two fractions was measured. More than 90% of the activity was found in the particulated fractions, and 94% of NAD-glutamate dehydrogenase, a typical soluble enzyme, was recovered in the particulated fractions, and 94% of NAD-glutamate dehydrogenase, a typical soluble enzyme, was recovered in the particulated fractions.

***Molecular Mass and Subunit Structure***—After gel filtration using Superdex G-200 prep grade at the final step of the purification, the dye-linked d-proline dehydrogenase activity was eluted as one main peak, and the elution curve of activity practically coincided with that of the protein elution curve (Fig. LA). The molecular mass of the enzyme corresponded to about 145 kDa (Fig. 1B). The molecular mass of the subunit was estimated by SDS-gradient/PAGE. The SDS-gradient/PAGE of the enzyme showed only one major band; the enzyme consists of an identical subunit (Fig. 2). The molecular mass of the subunit was estimated to be about 42 kDa from the standard curve obtained from SDS-gradient/PAGE. This indicates that the enzyme may be composed of a tetrameric structure.

Stability—The thermostability of the enzyme was examined. The enzyme retained its full activity after incubation at temperatures from 30 to 80 °C for 10 min but remarkably lost its activity after incubation at 100 °C for 10 min (Fig. 3). When the enzyme was incubated between pH 4.0 and 10.0 at 50 °C for 10 min, the activity completely remained.

Effect of pH on the Enzyme Activity—The enzyme activity was measured at various pHs in 200 mM buffer. Potassium phosphate, HEPES, Tris/HCl and glycine/KOH buffers were used for the assays at pH 6.5–8.0, pH 7.0–8.5, pH 7.5–8.5, and pH 9.0–10.0, respectively. The maximum activity was observed around pH 7.5 for the d-proline dehydrogenation, and the activity was observed at pH 7.5 and 8.5.

Effect of Temperature on the Enzyme Activity—The enzyme activity was measured at various temperatures (40–70 °C) in 200 mM Tris/HCl buffer (pH 8.0). The assay was started by the addition of Cl₂Ind after preincubation for 2 min at various temperatures. The enzyme activity was linearly increased with increasing temperature to 70 °C. The highest activity was above 70 °C, and the correct assay was not achieved above 70 °C, because of the non-enzymatic decolorization of Cl₂Ind.

Substrate and Electron Acceptor Specificity—The ability of
Prosthetic Group—The flavin compound extracted from the purified enzyme with 1% PCA was analyzed by HPLC. The flavin compound in the enzyme extract was identified to be FAD and not FMN.

N-terminal Amino Acid Sequence—The N-terminal sequence of the enzyme was analyzed by an automated Edman degradation protein sequencer. The N-terminal amino acid sequence of the subunit (28-amino acid residues) was determined to be MKVAIVGGIGLFTAYHLRQQGADVVI.

Cloning and Sequencing of Dye-linked D-Proline Dehydrogenase Gene—For cloning of the dye-linked d-proline dehydrogenase, the XbaI genomic library of Pb. islandicum was screened using 32P-labeled oligonucleotide mixtures as the probe. After screening of recombinant plasmids by Southern hybridization, positive clones were selected, and pDPDH1 carrying a 4.0-kbp XbaI fragment from the genome of Pb. islandicum was obtained from one clone. Fig. 4 shows the restriction map of the 4.0-kbp insert DNA from the recombinant pDPDH1. From the further Southern hybridization analysis, a dye-linked d-proline dehydrogenase coding sequence was determined to exist in the SalI fragment in the inserted DNA of pDPDH1 (Fig. 4). The 1.9-kbp SalI fragment was subcloned and sequenced (pDPDH2). We found a 1089-bp single open reading frame encoding 363 amino acids (Fig. 5). The deduced N-terminal amino acid sequence was identical to that obtained from protein sequencing (underlined in Fig. 5). The transcription product showed a molecular mass of 40,341 Da. In the upstream of the start codon in the enzyme gene, a sequence TATAAA like a bacterial promoter region was recognized (indicated by asterisks in Fig. 5).

Amino Acid Sequence Alignment of Dye-linked D-Proline Dehydrogenase—Similarity in the amino acid sequence between dye-linked d-proline dehydrogenase and other enzymes was searched using the BLAST server in GenBankTM data bases. The Pb. islandicum dye-linked d-proline dehydrogenase exhibited similarity to those of proteins containing a putative one in S. solfataricus (NCBI accession number AE006718:91–1188) (38% identity) (15), a hypothetical one in Sulfolobus tokodaii strain 7 (NCBI accession number AP000987:24952–25999) (36%) (16), a probable oxidoreductase in F. aeruginosa (NCBI accession number AE004921:3644–4894) (23%) (17), and a d-amino acid dehydrogenase small subunit in E. coli O 157:H7 (NCBI accession number AE005336:212–1510) (25%) (18).

Expression of Dye-linked D-Proline Dehydrogenase Gene—We prepared the transformant E. coli pDPDH3, which produced dye-linked d-proline dehydrogenase. Almost all of the enzyme activity was detected in the precipitate fraction by centrifugation (10,000 × g for 10 min) after disruption of the transformant E. coli cells. Thus, we disrupted the cells suspended in

**Dye-linked d-Proline Dehydrogenase from a Hyperthermophilic Archaeon**

**TABLE II**

| Substrate                  | Relative activity |
|---------------------------|------------------|
| d-Proline                 | 100              |
| allo-4-Hydroxy-d-proline  | 89               |
| d-Isoleucine              | 49               |
| d-Valine                  | 41               |
| d-Leucine                 | 39               |
| d-Histidine               | 30               |
| d-Phenylalanine           | 28               |
| d-Alanine                 | 26               |
| d-Glutamate               | 23               |
| d-Aspartate               | 21               |
| d-Threonine               | 15               |
| d-Tryptophan              | 14               |
| d-Serine                  | 13               |
| d-Arginine                | 10               |
| L-Proline                 | 0                |

**Fig. 3. Thermostatbility of dye-linked d-proline dehydrogenase.** The enzyme (pH 7.2) was incubated at each temperature, and the residual activity was determined by the standard assay method for 10 min at 50 °C.

**Fig. 2. SDS-PAGE of the enzyme.** Left column, molecular maker proteins; right column, the enzyme. A gradient gel (2–15%) was used.
Tris/HCl buffer (pH 8.0) supplemented with 1% Tween 20 to recover the activity in the supernatant solution after centrifugation. The enzyme was purified to homogeneity by heat treatment and two column chromatographies (Table III). An efficient purification of the enzyme was achieved; about 6.5 mg of the pure enzyme was prepared from the crude extract of E. coli cells obtained from a 500 ml culture medium. The molecular mass of the recombinant enzyme (about 42 kDa) was the same as that of the native enzyme judging from the SDS-gradient/PAGE. The N-terminal amino acid sequence is identical to that determined with the enzyme purified from Pb. islandicum cells.

**DISCUSSION**

In this study, we have found the activity of dye-linked D-proline dehydrogenase in the membrane fraction of a terrestrial archaeon. The probal promoter region is indicated by asterisks. The bold underlined sequence was determined from the N-terminal region of Pb. islandicum dye-linked D-proline dehydrogenase. The underlined sequence was the SalI region. The asterisks below the amino acid sequence is the FAD-binding motif.

**TABLE III**

| Step | Total protein | Total activity | Specific activity | Yield |
|------|---------------|----------------|------------------|-------|
| mg   | units         | units/mg       | %                |       |
| Crude extract | 1745 | 515 | 0.29 | 100 |
| Heat shock | 260 | 310 | 1.19 | 60 |
| DEAE-Toyopearl | 102 | 178 | 1.73 | 35 |
| Q-Sepharose | 6.46 | 35.1 | 13.2 | 16 |

**FIG. 4.** Restriction map for 4.0-kbp SalI fragment in pDPDH1. The thick arrow represents the dye-linked D-proline dehydrogenase coding sequence. The 1.9-kbp SalI fragment (bold dashed line) containing the coding region was subcloned and sequenced.

**FIG. 5.** Nucleotide sequence of the SalI fragment subcloned from pDPDH1 and the deduced amino acid sequence of Pb. islandicum dye-linked D-proline dehydrogenase. The probable promoter region is indicated by asterisks. The bold underlined sequence was determined from the N-terminal region of Pb. islandicum dye-linked D-proline dehydrogenase. The underlined sequence was the SalI region. The underlined sequence below the amino acid sequence is the FAD-binding motif.
Dye-linked d-Proline Dehydrogenase from a Hyperthermophilic Archaeon

Ph. islandicum JCM 9189.

The most preferable substrate for this enzyme was d-proline and not d-alanine. The presence of d-alanine dehydrogenase as dye-linked d-amino acid dehydrogenase has been reported so far in some mesophilic bacteria such as P. aeruginosa (3) and E. coli (6) but not the presence of dye-linked d-proline dehydrogenase.

The reactivity of d-proline for P. aeruginosa enzyme is about one-fifth that of d-alanine (3). Thus, the enzyme in Ph. islandicum is largely different from d-proline dehydrogenase reported so far in this respect. To our knowledge, this is the first example showing the existence of dye-linked d-proline dehydrogenase as a dye-linked amino acid dehydrogenase.

The product from d-proline by the enzyme reaction was 1-pyrroline-5-carboxylate. We have already found the dye-linked l-proline dehydrogenase in the hyperthermophilic archaeon Thermococcus profundus and identified the l-proline-5-carboxylate as a product of the enzyme reaction from l-proline (19). This indicates that the regiospecificity of dye-linked d-proline dehydrogenase is different from that of the dye-linked l-proline dehydrogenase as well as the difference in stereoselectivity for proline. In addition, the substrate specificity is comparatively low; various d-α-amino acids containing d-isoleucine and d-valine as well as d-proline could be the electron donor. In contrast, the T. profundus dye-linked l-proline dehydrogenase acts on the l-configuration of proline, and its substrate specificity is extremely high (19). Thus, from the aspect of specificity, the dye-linked d-proline dehydrogenase is totally different from the dye-linked l-proline dehydrogenase, although both are similar dye-linked FAD-dependent amino acid dehydrogenases. A detailed comparison of the structure and function of the two dehydrogenases may give us further information about the novel membrane-bound dye-linked amino acid dehydrogenases. The three-dimensional analysis of the enzyme is now under investigation.

The sequence similarity of the enzyme was detected from the data research on genome sequences, and a high similarity (about 38%) in amino acid sequence was detected in the sequence of a putative oxidoreductase in the aerobic hyperthermophilic archaeon S. solfataricus (15) and the S. tokodaii strain 7 (16). Both Sulfolobus strains belong to Crenarchaeota similar to Ph. islandicum. In addition, we compared the sequence of Ph. islandicum dye-linked d-proline dehydrogenase with those of other mesophilic d-amino acid dehydrogenases (Fig. 6). The Ph. islandicum dye-linked d-proline dehydrogenase has a low similarity to a dye-linked d-amino acid dehydrogenase small subunit from bacteria including Klebsiella pneumoniae (25%) (20) and homologues from several bacteria, including E. coli O157 (25% similarity) (17) and Mesorhizobium loti (22%) (21). In contrast, a partially high sequence homology was detected in the N-terminal and C-terminal regions between the Ph. islandicum enzyme and bacterial d-amino acid dehydrogenases. In the case of mesophilic d-amino acid dehydrogenases, the N-terminal region is considered to be a site anchoring it to

FIG. 6. Multiple sequence alignment of Ph. islandicum d-proline dehydrogenase and other bacterial d-amino acid dehydrogenases. Highly conserved residues are depicted in black boxes and the less strongly conserved in gray boxes. Proteins shown on figure: K. aerogenes, d-amino acid dehydrogenase from K. aerogenes (NCBI accession number AE016253:331–1629); V. cholerae, d-amino acid dehydrogenase homologue from Vibrio cholerae (NCBI accession number AE004164:1999–3264); X. fastidiosa, d-amino acid dehydrogenase homologue from Xylella fastidiosa (NCBI accession number AE003925:86–1377); E. coli, d-amino acid dehydrogenase homologue from E. coli O157 H7; N. meningitidis, d-amino acid dehydrogenase homologue from Neisseria meningitidis (NCBI accession number AE002375:121–1377); M. loti, d-amino acid dehydrogenase homologue from Mesorhizobium loti P90301:179600–180838; S. solfataricus, the probable oxidoreductase from S. solfataricus strain P2; and S. tokodaii, the hypothetical protein from S. tokodaii. In the case of the S. tokodaii protein sequence, a 58-amino acid sequence in the N-terminal region was added to that obtained from the NCBI data base. Kawai et al. (16) assigned ATG as the start codon for the hypothetical protein in the S. tokodaii gene, because the criterion used for the assignment of the potential coding region in the S. tokodaii strain 7 genomic sequence was the identification of the sense codons starting with ATG or GTG. However, the result of the alignment of the Ph. islandicum dye-linked d-proline dehydrogenase shows that the sense codon of the enzyme gene may start with minor TTG codon, which is frequently used for the start codon in Cyanobacteria (24).
the membrane (22). This indicates that the N-terminal region is postulated to be one of the anchoring sites to the membrane. However, most parts of the sequence of *Pb. islandicum* enzyme except for N-terminal and C-terminal regions are fairly different from those of d-amino acid dehydrogenases (Fig. 6). This suggests that the dye-linked d-proline dehydrogenase is inherently different from bacterial d-amino acid dehydrogenases so far reported and is a novel type of FAD-dependent d-amino acid dehydrogenase.

In general, dye-linked dehydrogenases have high potential utilization as a specific element for biosensors (23). The highly thermostable dye-linked d-proline dehydrogenase exhibits a high potential usefulness for application to the d-amino acid analysis.

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