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ORIGINAL PAPER

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Gene and primary structures of dye-linked L-proline dehydrogenase from the hyperthermophilic archaeon *Thermococcus profundus* show the presence of a novel heterotetrameric amino acid dehydrogenase complex

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Abstract Dye-linked L-proline dehydrogenase catalyzes the oxidation of L-proline in the presence of artificial electron acceptors such as 2, 6-dichloroindophenol and ferricyanide. The enzyme from the hyperthermophilic archaeon *Thermococcus profundus* was purified and characterized for the first time in archaea by Sakuraba et al. in 2001. In this study, cloning and sequencing analyses of the gene encoding the enzyme and functional analysis of the subunits were performed. The gene formed an operon that consisted of four genes, *pdh*A, *pdh*B, *pdh*F, and *pdh*X, which are tandemly arranged in the order of *pdh*A-F-X-B. SDS-PAGE analysis of the purified recombinant enzyme showed four different bands corresponding to α (54 kDa), β (43 kDa), γ (19 kDa), and δ (8 kDa) subunits encoded by *pdh*A, *pdh*B, *pdh*F, and *pdh*X, respectively, and the molecular ratio of these subunits was determined to be equal. This indicates that the enzyme consists of a heterotetrameric $\alpha\beta\gamma\delta$ structure. Functional analysis of each subunit revealed that the β subunit catalyzed the dye-linked L-proline dehydrogenase reaction by itself and that, unexpectedly, the α subunit exhibited dye-linked NADH dehydrogenase activity. This is the first example showing the existence of a bifunctional dye-linked L-proline/NADH dehydrogenase complex. On the basis of genome analysis, similar gene clusters were observed in the genomes of *Pyrococcus horikoshii, Pyrococcus abyssi, Pyrococcus furiosus*, and *Archaeoglobus fulgidus*. These results indicate that the dye-linked L-proline dehydrogenase is a novel type of heterotetrameric amino acid dehydrogenase that might be widely distributed in the hyperthermophilic archaeal strain.

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Introduction

Dye-linked L-proline dehydrogenase (dye-L-proDH) catalyzes the oxidation of L-proline in the presence of artificial electron acceptors such as 2, 6-dichloroindophenol (Cl2Ind) and ferricyanide. The presence of dye-L-proDH has been reported in *Escherichia coli* and *Salmonella typhimurium* cells, and some fundamental properties of these enzymes have been investigated (Scarplla and Soffer 1978; Menzel and Roth 1981a, 1981b; Graham et al. 1984; Maloy 1987). However, information on the detailed structure and function of the enzyme is still lacking because of its low stability.

Recently, we reported the presence of a dye-L-proDH in the hyperthermophilic archaeon *Thermococcus profundus* DSM9503 (Sakuraba et al. 2001). This is the first report showing the occurrence of a dye-L-proDH in hyperthermophilic archaea. The purified enzyme showed the highest thermostability among dye-dependent amino acid dehydrogenases so far found. The archaeal enzyme was shown to be significantly different from those of the *E. coli* and *S. typhimurium* enzymes in many respects of the catalytic and molecular properties (Allen et al. 1993; Xia et al. 1995). This indicates that the archaeal enzyme may be a novel type of dye-L-proDH. In this study, we determined the primary structure of the dye-L-proDH from *T. profundus* and the genetic organization of the encoding genes. The results showed that the enzyme consists of four different subunits, α, β, γ, and δ. In addition, the functional and gene structural analyses indicated that the enzyme is a novel type of bifunctional amino acid dehydrogenase that exhibited NADH dehydrogenase activity as well as L-proline dehydrogenase

activity. We describe here the molecular and structural characteristics of the novel type of heterotetrameric amino acid dehydrogenase.

Materials and methods

Materials

pUC18, pET15b, and TKR MEGALABEL were purchased from TaKaRa (Kyoto, Japan). The [γ-^{32}P]ATP and Probe Quant G-50 column were purchased from Amersham Pharmacia Biotech (Tokyo, Japan). *Escherichia coli* strain JM 109 and BL21-CodonPlus(DE3)-RIL cells were purchased from STRATAGENE (Tokyo). Restriction enzymes were purchased from New England Biolabs (Tokyo) and TaKaRa. The other chemicals were analytical-grade reagents from nacalai tesque (Kyoto).

Cloning and sequencing of the enzyme gene

To obtain a clone containing the dye-L-proDH gene, *Thermococcus profundus* chromosomal DNA was prepared by the method described previously (Murray and Thompson 1980), digested with several restriction enzymes, and then separated by 0.8% agarose gel electrophoresis. The degenerate oligonucleotides, 5′-AT(T, C, A)CA(T, C)TT(T, C)GA(A, G)GG(T, C, A, G)CAGCC-3′, were designed as the DNA probe for the *T. profundus* dye-L-proDH gene based on the amino acid sequence of IHFEGQP, which has been determined to be a part of the N-terminal amino acid sequence of the α subunit of the natural dye-L-proDH (Sakuraba et al. 2001). A highly specific activity probe was generated using 1.5 pmol of this oligonucleotide mixture, 10 U of T4 polynucleotide kinase, and 1.85 MBq of [γ-^{32}P]ATP. The separated DNA fragments on agarose gel were subjected to Southern blotting with the ^{32}P-labeled probe. An approximately 6-kb *Pst*I fragment gave a positive signal on Southern hybridization. The *Pst*I fragment from the chromosomal DNA was inserted into the *Pst*I site of plasmid pUC18 and then *E. coli* JM109 cells were transformed. The transformants were selected on a Luria-Bertani (LB) plate containing ampicillin (0.005%), isopropyl-β-D-thiogalactopyranoside (IPTG) (0.01%), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (0.02%). The colonies were transferred and fixed on nylon membranes. After screening of the recombinant plasmids by Southern hybridization, a positive plasmid containing the 5.8-kb *Pst*I fragment, pUPDH19, was isolated and used as the template for DNA sequencing. The sequencing was performed using the dideoxynucleotide chain-termination method with an Applied Biosystems PRISM Model 377 DNA sequencer. The sequence data were analyzed using GENETYX-SV/RC 9.0 software (Software Development, Tokyo). The sequence data reported in this paper have been submitted to DDBJ under accession number AB055743.

Expression and purification of the recombinant dye-L-proDH complex

The pUPDH19 was digested with *Nru*I and ligated to produce an expression vector, pUPDH. The *E. coli* strain JM109 cells were transformed with pUPDH. The transformants were grown at 37°C in SB medium (1.2% tryptone peptone, 2.4% yeast extract, 1.25% K_2HPO_4, 0.38% KH_2PO_4, and 0.5% glycerol) containing ampicillin and IPTG. After a 12-h cultivation, cells were collected and suspended in buffer A [10 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA] and disrupted by sonication. After centrifugation (15,000 *g*, 15 min), the solution was heated at 70°C for 10 min. After centrifugation (15,000 *g*, 10 min), the supernatant solution was brought up to 20% saturation with $(NH_4)_2SO_4$. The enzyme solution was applied on a Butyl-Toyopearl (Tosoh, Tokyo) column that had been previously equilibrated with buffer A supplemented with 20% $(NH_4)_2SO_4$. After the column was washed with the same buffer, the enzyme was eluted with a linear gradient of 20–0% $(NH_4)_2SO_4$ in buffer A. The active fractions were pooled and dialyzed against buffer A. The enzyme solution was applied on a Red-Sepharose CL-4B column that had been previously equilibrated with buffer A, and the enzyme was eluted with a linear gradient of 0–1.0 M NaCl in the same buffer. The active fractions were pooled, dialyzed against buffer A, and used as the purified enzyme preparation.

Expression and purification of each subunit

Dye-L-proDH forms a complex that consists of four different subunits with the molecular masses of 52, 43, 19, and 11 kDa. The enzyme genes encoding the 52-, 43-, 19-, and 11-kDa subunits were designated *pdh*A, *pdh*B, *pdh*F, and *pdh*X, respectively. The pUPDH was used as the template, and the following sets of oligonucleotides were used as PCR primers to construct of the expression vectors for these genes: 5′-ATTACATATGCGCCTCACTGAACATCCC-ATT-3′ (*pdh*A-*Nde*I) and 5′-TATGGATCCAACGTCAGGCA-TATCCATCG-3′ (*pdh*A-*Bam*HI) for *pdh*A; 5′-ATTACATATGCCTGACGTTCCCTCCTA-3′ (*pdh*F-*Nde*I) and 5′-TATGGATCCTTTGGATCTTCAGTCATT-3′ (*pdh*F-*Bam*HI) for *pdh*F; 5′-ATTACATATGACTGAAGATCCAAAGGG-3′ (*pdh*X-*Nde*I) and 5′-TATGGATCCTTTTGGCTTC-ACTCCTCATC-3′ (*pdh*X-*Bam*HI) for *pdh*X; and 5′-ATTA-CATATGAGGAGTGAAGCCAAAAC-3′ (*pdh*B-*Nde*I) and 5′-TATGGATCCAAATTCAGCCCATCTGAAGTGC C TGA-3′ (*pdh*B-*Bam*HI) for *pdh*B. In each case, the forward primer introduced a unique *Nde*I restriction site overlapping the 5′-initiation codon, and the reverse primer introduced a unique *Bam*HI restriction site proximally to the 3′-end of the termination codon. The amplified fragments were digested with *Nde*I and *Bam*HI and ligated with the expression vector pET15b linearized with *Nde*I and *Bam*HI to generate pET15b/*pdh*A, pET15b/*pdh*F, pET15b/*pdh*X, and pET15b/*pdh*B. Expression and purification of each subunit were performed with the following procedure. The *E. coli* strain BL 21-CodonPlus(DE3)-RIL cells were transformed with each vector. The transformants were grown for 4 h at 37°C in SB medium in the presence of ampicillin (0.005%). The IPTG was added to 0.1 mM and cultivation was continued for 18 h at 18°C. The cells were collected by centrifugation and suspended in 10 mM Tris/HCl (pH 7.5) and disrupted by sonication. After centrifugation, imidazole and NaCl were added to the supernatant up to 50 mM and 0.5 M, respectively. The solution was applied on a HiTrap nickel-charged chelating column (Amersham Biosciences) equilibrated with 10 mM Tris/HCl (pH 7.5) containing 50 mM imidazole and 0.5 M NaCl. The column was washed with the same buffer, and the enzyme was eluted with 10 mM Tris/HCl (pH 7.5) containing 0.5 M imidazole and 0.5 M NaCl. Active fractions were pooled and the solution was used for various experiments. For enzyme purification, all buffers were degassed before use.

Enzyme assay

Dye-L-proDH activity was assayed by measuring the reduction rate of Cl2Ind as previously described (Sakuraba et al. 2001). Dye-linked NADH dehydrogenase (dye-NADHDH) activity was assayed by measuring the decrease in Cl2Ind at 600 nm in the presence of NADH. The reaction mixture was the same as that for the dye-L-proDH activity, except that L-proline was altered by NADH (0.1 mM) and FAD (0.05 mM) was added. The non-enzymatic decrease in Cl2Ind in the presence of NADH at 50°C was monitored in the absence of enzyme.

Molecular mass determination and polyacrylamide gel electrophoresis

The molecular mass of the native enzyme was determined by gel filtration column chromatography using Superose 6 HR

(Amersham Pharmacia Biotech). Thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), and ribonuclease A (13.7 kDa) were used as the molecular standards (Amersham Pharmacia Biotech). Subunit molecular mass was determined by SDS-PAGE, which was carried out with 15% polyacrylamide gel according to the method of Lämmli (1970). Maltose-binding protein (MBP)-β-galactosidase (175 kDa), MBP-paramyosin (83 kDa), glutamate dehydrogenase (62 kDa), aldolase (47.5 kDa), triosephosphate isomerase (32.5 kDa), β-lactoglobulin (25 kDa), lysozyme (16.5 kDa), and aprotinin (6.5 kDa) were used as the molecular standards (New England Biolabs). Native PAGE was carried out with 7.5% polyacrylamide gel according to the method of Davis (1964). Activity staining was performed as previously described (Sakuraba et al. 2001).

Stereospecificity of hydrogen transfer

The stereospecificity of hydrogen transfer of NADH catalyzed by the dye-linked L-proline/NADH dehydrogenase complex was studied by the ^{1}H NMR method. [4*R*-^{2}H] NADH was produced by the method described previously (Gassner et al. 1994). [4*R*-^{2}H] NADH (about 3 μmol) was incubated at 50°C for 30 min with the enzyme in 200 mM Tris/HCl (pH 7.5) and 3 μmol of Cl2Ind. The ^{1}H NMR spectra of the aromatic region of the nicotinamide ring of NAD produced were measured on a 400-MHz NMR apparatus (JEOL, Japan).

Determination of flavin, non-heme iron, and absorption spectra

Flavin compound was extracted from the subunit protein with 1% perchloric acid, and the precipitate formed was removed by centrifugation. The supernatant was used to identify the flavin compound by HPLC system with a TSKgel ODS-80Ts (Tosoh, Japan). The elution was operated by a flow rate of 1.0 ml/min as follows: for 10 min of isocratic elution with 10 mM potassium phosphate (pH 6.0), 25 min of linear gradient to 50% methanol, and 10 min of linear gradient to 100% methanol. The elution peak was monitored by the absorbance at 260 nm. FAD content was determined spectrophotometrically as previously described (Cafaro et al. 2002). After solutions of the enzyme complex, α subunit and β subunit were boiled for 10 min 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. Non-heme iron was determined by the method of Fish (1988). Absorption spectra were recorded using a GenespecIII spectrophotometer (Hitachi, Japan).

N-terminal amino acid sequence analysis

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, Japan).

Genome databases

To search genome databases, the following URLs were used: *Pyrococcus abyssi* database: http://www.genoscope.cns.fr/; *Pyrococcus horikoshii* database: http://www.bio.nite.go.jp/; and *Archaeoglobus fulgidus* database: http://www.tigr.org/. The genome information of *Pyrococcus furiosus* is available in the Kyoto Encyclopedia of Genes and Genomes (KEGG): http://www.genome.ad.jp/kegg/.

Results

Analysis of the enzyme genes

Sequencing of pUPDH19 gave the complete sequences of *pdh*A, *pdh*B, *pdh*F, and *pdh*X genes with a total length of 3,399 bp (Fig. 1). The 3′ part of *pdh*A, *pdh*F, and *pdh*X was slightly overlapped by the 5′ part of *pdh*F, *pdh*X, and *pdh*B, respectively, in the order of *pdh*A-F-X-B. *pdh*A (1,449 bp) and *pdh*B (1,155 bp) encoded 483 and 385 amino acids with molecular weights of 51,921 and 42,682, respectively. *pdh*F (516 bp) and *pdh*X (294 bp) encoded 172 and 98 amino acids with the molecular weights of 18,826 and 10,642, respectively. Upstream of the start codon in the *pdh*A gene, a sequence AAATCCTCATAAA like an archaeal promoter region was recognized (underlined in Fig. 1).

Subunit structure and molecular mass

Purification of the recombinant dye-L-proDH from recombinant *Escherichia coli* is summarized in Table 1. The purified enzyme was found to be homogeneous; the native PAGE of the enzyme gave a single band (Fig. 2A), which corresponded to active staining. Upon SDS-PAGE analysis, two distinct bands with molecular masses of about 54 (α) and 43 kDa (β), along with two minor 19- (γ) and 8-kDa (δ) bands, were observed (Fig. 2B). The N-terminal amino acid sequences of the 54- and 43-kDa proteins were determined to be MRLTEHPILDFSERRGR and MRSEAKTVIIGGGIIGLSIAYN, respectively. The sequences of 54- and 43-kDa proteins coincided with those of the α and β subunits deduced from the respective gene sequences. On the other hand, the N-terminal sequences of the 19- and 8-kDa proteins were determined to be PDVPSYLRRGYITPEELFGM and MTEDPKGKIII, respectively, and these sequences coincided with those deduced from the *pdh*F and *pdh*X gene sequences, respectively. After SDS-PAGE, the stained gel was scanned and the relative ratio of the peak areas was determined to be 1.30 (α):1.00 (β):0.39 (γ):0.22 (δ) using NIH Image software. The molar ratio of α:β:γ:δ was calculated to be about 1:1:1:1 when taking into account their molecular weights. The molecular mass of the enzyme was determined by Superose 6 gel filtration chromatography and estimated to be about 120 kDa.

Enzymological characteristics of the recombinant enzymes

About 90% of the activity remained after incubation at 70°C for 10 min, and in the region of pH 4.5–9.0, the enzyme was not inactivated (data not shown). The optimum temperature, pH, and K_m value for L-proline were determined to be 85°C, 7.5, and 2.50 mM,

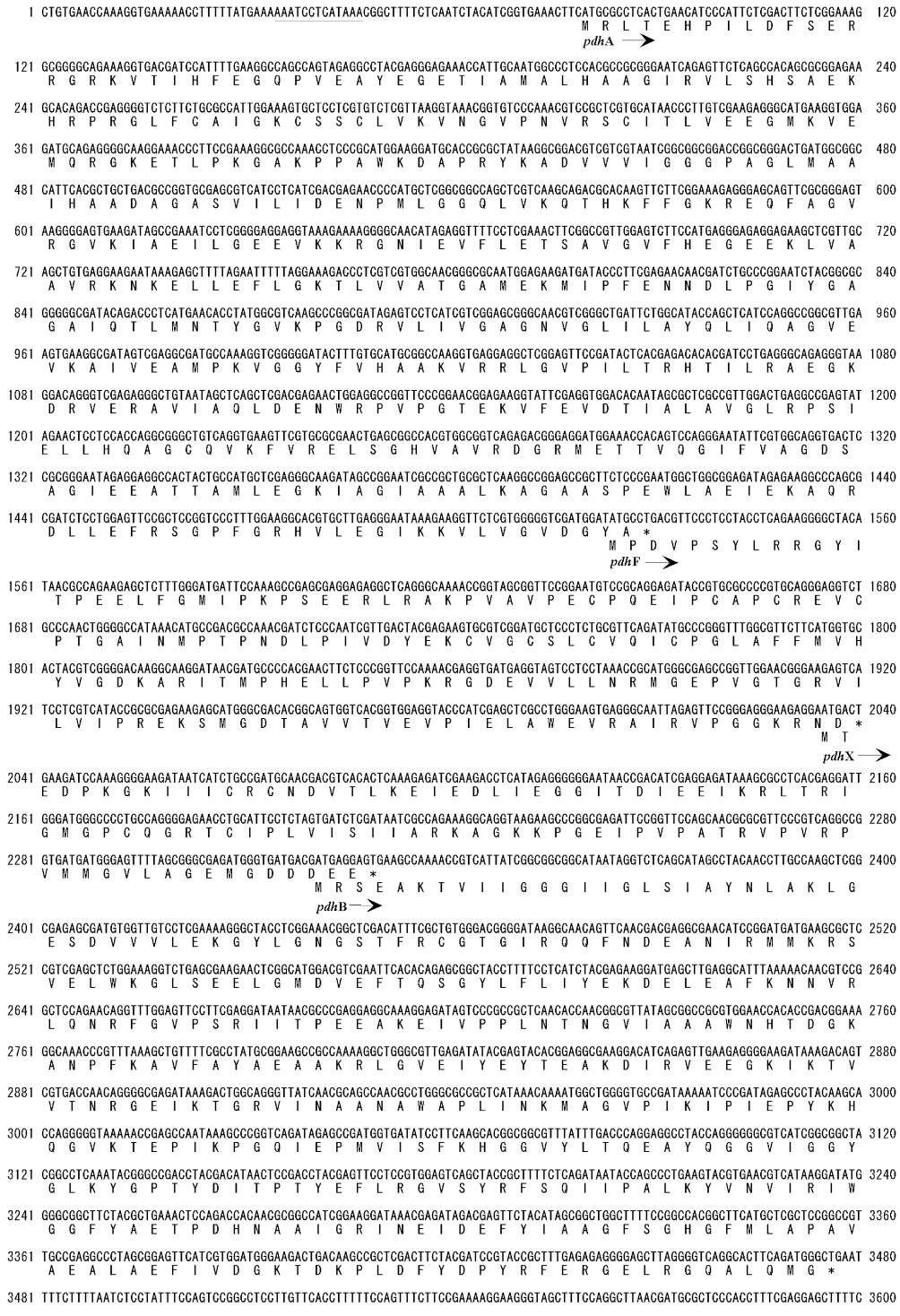

Fig. 1 Nucleotide and deduced amino acid sequences of 3.6-kbp DNA fragment containing *pdh*A, *pdh*F, *pdh*X, and *pdh*B genes from *Thermococcus profundus*. 3′ End of *pdh*A and 5′-end of *pdh*F, 3′ end of *pdh*F and 5′ end of *pdh*X, and 3′ end of *pdh*X and 5′ end of *pdh*B are partially overlapped. A deduced promoter sequence is *underlined*

respectively (data not shown). The content of FAD was determined to be 1.96 mol of FAD per mol of enzyme complex.

Analysis of the amino acid sequence of each subunit

The deduced amino acid sequence of the α subunit from the nucleotide sequence showed high homologies with those of the *Pyrococcus abyssi* putative sarcosine oxidase α subunit (ORF ID, PAB0212, 75%), the *Pyrococcus horikoshii* putative D-nopaline dehydrogenase (PH1749, 73%), the *Pyrococcus furiosus* putative sarcosine oxidase α subunit (PF1795, 73%), and the *Archaeoglobus fulgidus* putative sarcosine oxidase

Table 1 Purification of recombinant dye-linked L-proline dehydrogenase from *Escherichia coli*

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Crude extract	518	1,240	0.42	100
Heat treatment	300	125	2.40	68
Butyl Toyopearl	111	14.4	7.71	14
Red Sepharose CL-4B	42.0	3.0	14.0	10

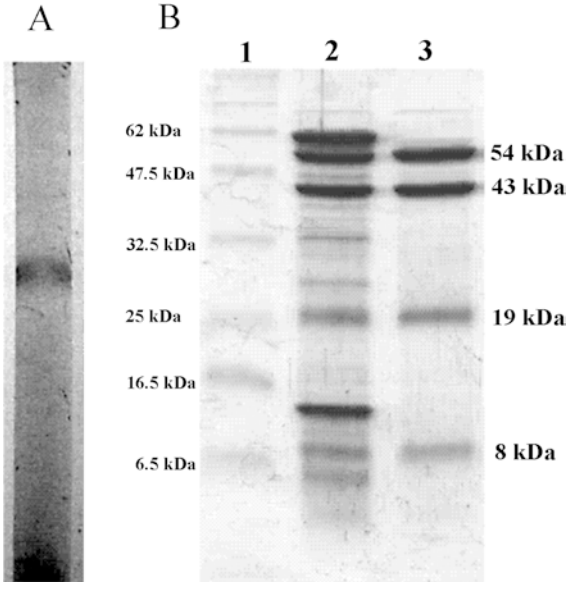

Fig. 2A, B Polyacrylamide gel electrophoresis analyses of the purified recombinant dye-linked L-proline dehydrogenase. **A** Native PAGE of the purified enzyme. **B** SDS-PAGE: lane 1, markers; lane 2, after Butyl-Toyopearl chromatography; lane 3, purified enzyme

α subunit (AF0273, 52%) (Fig. 3). In the two regions of 120–148 and 271–301 residues, the ADP-binding motif described by Wierenga et al. (1986) was observed in all sequences of these proteins. In addition, a [2Fe-2S] cluster motif was found in the region of 62–83 residues.

The β subunit exhibited an ADP-binding motif similar to the α subunit in the vicinity of the N-terminus. The amino acid sequence of the β subunit showed high homologies with those of the *P. furiosus* putative sarcosine oxidase β subunit (PF1798, 59%), the *P. horikoshii* putative sarcosine oxidase β subunit (PH1751, 56%), the *P. abyssi* putative sarcosine oxidase β subunit (PAB0214, 56%), and the *A. fulgidus* putative sarcosine oxidase β subunit (AF0274, 33%) (Fig. 4). In all cases, the ADP-binding motif was well conserved in the region of the N-termini.

The amino acid sequence of the γ subunit showed high homologies with those of *P. abyssi* putative polyferredoxin (PAB0213, 76%), *P. horikoshii* putative ferredoxin (PH1750, 76%), *P. furiosus* putative polyferredoxin (PF1796, 73%) (Fig. 5), and ferredoxins from various other organisms. Two [4Fe-4S] cluster motifs (CXXCXXCXXXCP) were observed in the two sequences of residues of 39–53 and 73–84. The two motifs are conserved in all the ferredoxin homologues.

The amino acid sequence of the δ subunit showed high homologies with those of *P. furiosus* D-nopaline dehydrogenase (PF1797, 74%) and *P. abyssi* hypothetical protein (PAB3086, 60%) (Fig. 6).

Functional analysis of each subunit of the enzyme complex

For characterization of the function of each subunit, the expressions of *pdh*A, *pdh*B, *pdh*F, and *pdh*X genes in *E. coli* were performed. The recombinant proteins were produced as the fusion proteins combined with the His-tagged sequence in their N-terminal regions. These subunits were highly purified using a HiTrap column.

The functions of each subunit are summarized in Table 2. Dye-L-proDH activity was determined using each of the subunits and detected only with the β subunit. Specific activities of the enzyme complex and the β subunit for dye-L-proDH activity were 14.0 and 3.9 U/mg, respectively. The spectrum of the β subunit showed the characteristics of a typical flavoprotein (Fig. 7B). The flavin compound extracted from the β subunit was identified as FAD. The content of FAD was determined to be 0.2 mol of FAD per mol of protein. We have attempted the detection of sarcosine oxidase and D-nopaline dehydrogenase but could not detect any activity of the two enzymes. On the other hand, the activity of dye-NADHDH was detected with the α subunit. This activity was also found with the enzyme complex. Specific activities of the enzyme complex and α subunit for dye-NADHDH activity were 0.68 and 0.18 μmol/min/mg, respectively. FAD was detected in the extract of the α subunit, but the content was considerably low (0.1 mol of FAD per mol of protein), like in the case of β subunit.

We analyzed the stereospecificity for hydrogen transfer of NADH catalyzed by the enzyme complex using [4*R*-^{2}H] NADH. When [4*R*-^{2}H] NADH was incubated with the enzyme in the presence of Cl2Ind, a resonance doublet around 8.7δ for the C-4 proton of NAD was not observed in the ^{1}H NMR spectrum (Fig. 8A). When NADH was used instead of [4*R*-^{2}H] NADH as the control, the doublet signal of the C-4 proton of NAD was observed in the NMR spectrum (Fig. 8B). This indicates that 4*R*-^{2}H of NADH remains and that 4*S*-^{1}H is removed by the enzyme reaction: the enzyme exhibits B-stereospecificity for the hydrogen transfer.

The presence of iron-sulfur cluster motifs was predictable on both the α and γ subunits from the information on the primary structure. To detect iron, we performed a specific staining for non-heme iron on the polyacrylamide gel (Brill et al. 1974; Leong et al. 1992). As a result, a specific band was detected in the gel. From

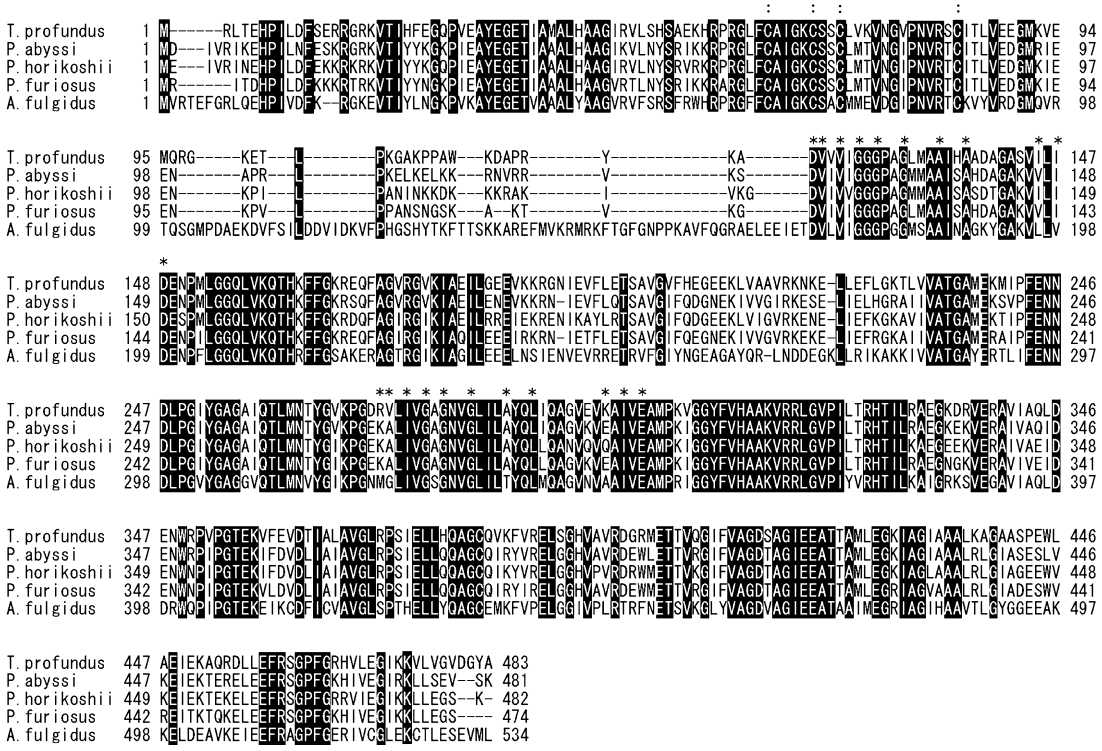

Fig. 3 Multiple sequence alignment of the α subunit and homologous sequences in archaea postulated from genome sequences. Putative proteins shown in the figure are follows: *P. abyssi*, sarcosine oxidase α subunit from *P. abyssi* (ORF ID, PAB0212); *P. horikoshii*, D-nopaline dehydrogenase from *P. horikoshii* (ORF ID, PH1749); *P. furiosus*, sarcosine oxidase α subunit from *P. furiosus* (ORF ID, PF1795); and *A. fulgidus*, sarcosine oxidase α subunit from *A. fulgidus* (ORF ID, AF0273). Conserved residues are depicted in *black boxes*. *Asterisks* and *double dots* represent the residues of ADP-binding motif described by Wierenga et al. (1986) and [2Fe-2S] iron-sulfur cluster motif, respectively

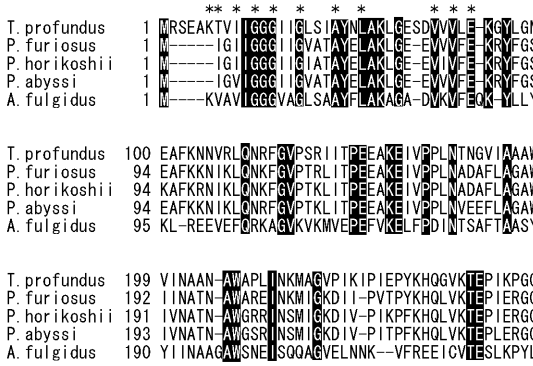

Fig. 4 Multiple sequence alignment of the β subunit and homologous sequences in archaea postulated from genome sequences. Putative proteins shown in the figure are the sarcosine oxidase β subunit from *P. furiosus* (ORF ID, PF1798), *P. horikoshii* (ORF ID, PH1751), *P. abyssi* (ORF ID, PAB0214), and *A. fulgidus* (ORF ID, AF0274), respectively. Conserved residues are depicted in *black boxes*. *Asterisks* represent the residues of ADP-binding motif described by Wierenga et al. (1986)

the spectrophotometric analysis of non-heme iron, the enzyme complex was determined to contain 8 mol of iron per mol of protein. The spectrum measurement of both the α and γ subunits indicated that iron-sulfur clusters are included (Fig. 7A, C). The α subunit was shown to contain the [2Fe-2S] iron-sulfur flavoprotein from its typical spectrum peaks at 340 and 450 nm. The γ subunit exhibited a broad peak at around 420 nm, which is a typical [8Fe-8S] ferredoxin (Bentrop et al. 1997; Menon et al. 1998).

Discussion

In a previous paper (Sakuraba et al. 2001), we reported catalytic properties and the presence of the

Fig. 5 Multiple sequence alignment of the γ subunit and homologous sequences in archaea postulated from genome sequences. Putative proteins shown in the figure are ferredoxin from *P. abyssi* (ORF ID, PAB0213), *P. horikoshii* (ORF ID, PH1750), *P. furiosus* (ORF ID, PF1796), and *A. fulgidus* (AF*pdh*F), which was located between AF0273 and AF0274, respectively. Conserved residues are depicted in *black boxes*. *Asterisks* represent the residues of iron-sulfur cluster motifs

novel dye-L-proDH in the hyperthermophilic archaeon *Thermococcus profundus* . In this study, we have shed light on the primary and subunit structures of the enzyme by gene cloning and sequencing and found that the enzyme consists of a unique complex of four different subunits.

Sequencing analysis of the dye-L-proDH gene showed that the gene coding dye-L-proDH forms an operon of four different genes of *pdh*A, *pdh*F, *pdh*X, and *pdh*B in this order. In the operon, the 3′ parts of *pdh*A, *pdh*F, and *pdh*X partially overlap the 5′ part of *pdh*F, *pdh*X, and *pdh*B, respectively (Fig. 1). In general, the expression of the distal gene in an operon is known to be dependent on the translation of the proximal gene, which has a stop codon overlapping or close to the initial codon of the distal gene. Here we found by SDS-PAGE analysis that the enzyme consisted of four different subunits (α, β, γ, and δ) with molecular masses of 54, 43, 19, and 8 kDa (Fig. 2B) and that their N-terminal amino acid sequences coincided with those deduced from the gene sequences. In addition, the molar ratio of these subunit constituents was calculated to be equal. In the previous report, we showed that the enzyme consists of α and β subunits judging from SDS-PAGE (Sakuraba et al. 2001). Therefore, we tried to purify the dye-L-proDH again from *T. profundus* cells. As a result, four bands corresponding with α, β, γ, and δ subunits were observed by SDS-PAGE. Both bands of two smaller subunits (γ and δ) were rather broad and obscure compared with those of the larger α and β subunits on an SDS-PAGE plate. That seems to lead to misunderstanding about the subunit structure. In addition, we reported a molecular mass of about 160 kDa for the native enzyme with the gradient PAGE in the previous paper. In this study, we examined the molecular mass of the recombinant enzyme using another method of gel filtration and determined it to be 120 kDa. From these results, we proposed that the dye-L-proDH from *T. profundus* consists of a complex of a heterotetrameric $\alpha\beta\gamma\delta$-structure. The enzymological properties of the recombinant enzyme are similar to those of the native enzyme. The only difference between the recombinant and native enzymes was observed in their specific activities: the specific activity of the former enzyme is 4.5 times higher than that of the latter. In the previous purification procedure of the native enzyme, the buffers of higher pHs, such as Tris/HCl, pH 9.0 and 8.0, are used in the last two steps of UnoQ

Fig. 6 Multiple sequence alignment of the δ subunit and homologous sequences in archaea postulated from genome sequences. Proteins shown in the figure are follows: *P. furiosus*, putative D-nopaline dehydrogenase from *P. furiosus* (ORF ID, PF1797); *P. horikoshii*, unknown protein from *P. horikoshii* (PH*pdh*X) that was located between PH1750 and PH1751; *P. abyssi*, hypothetical protein from *P. abyssi* (ORF ID, PAB3086); and *A. fulgidus*, unknown protein from *A. fulgidus* (AF*pdh*X) that was located between AF*pdh*F and AF0274. Conserved residues are depicted in *black boxes*

Table 2 Predicted functions of each subunit and the specific activities of the dye-linked L-proline dehydrogenase and the dye-linked NADH dehydrogenase of the enzyme complex and each subunit

	Predicted function	Practical function	Specific activities of dye-linked dehydrogenase for:	
			L-proline (U/mg)	NADH (μmol/min/mg)
Complex			14	0.68
α subunit	Sarcosine oxidase α subunit	Dye-linked NADH dehydrogenase	–	0.18
β subunit	Sarcosine oxidase β subunit	Dye-linked L-proline dehydrogenase	3.9	–
γ subunit	Ferredoxin	Ferredoxin	–	–
δ subunit	Unknown protein	Unknown protein	–	–

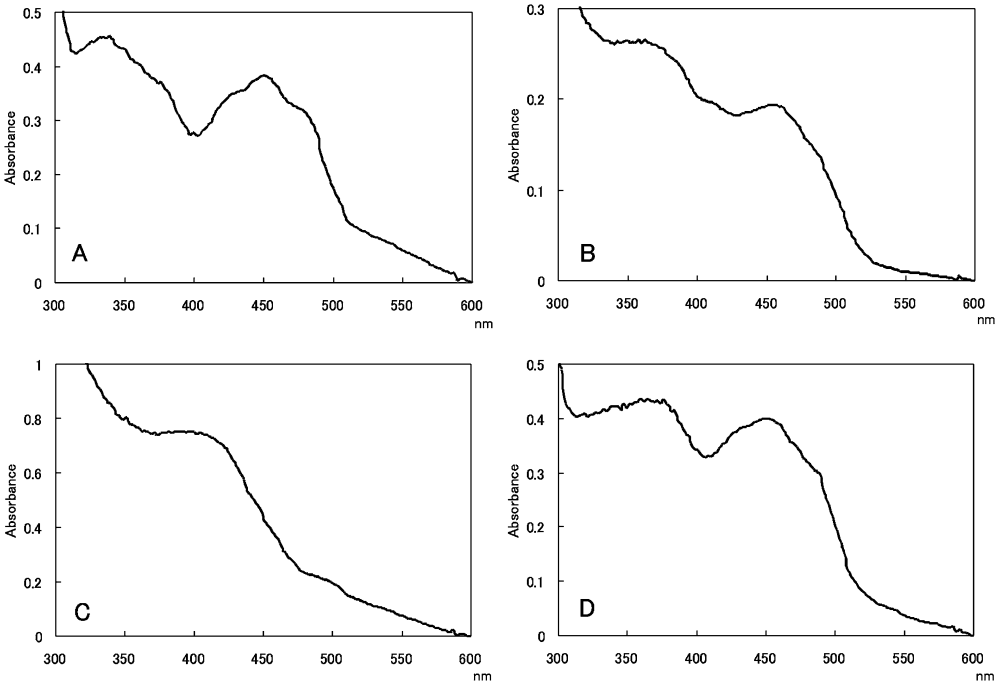

Fig. 7A–D Absorption spectra of α, β, γ, and enzyme complex. **A**, **B**, and **C** are the α, β and γ subunits, respectively, in 10 mM Tris/HCl (pH 7.5) containing 0.5 M imidazole and 0.5 M NaCl. **D** Enzyme complex in 10 mM Tris/HCl (pH 7.5). Protein concentrations are α, 5.9 mg/ml; β, 4.2 mg/ml; γ, 3.0 mg/ml; and enzyme complex, 1.7 mg/ml

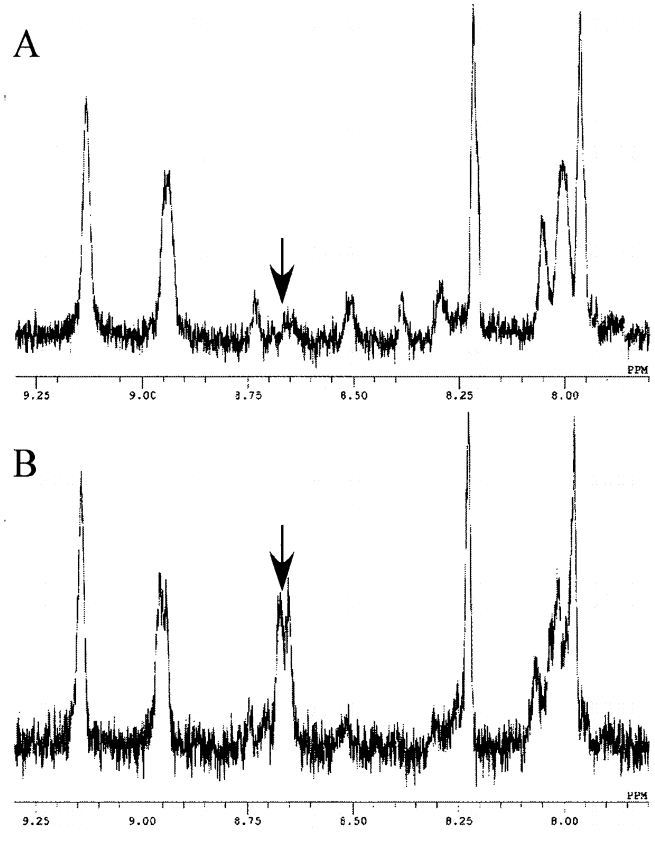

Fig. 8 ^{1}H-NMR spectra of the aromatic region of NAD after incubation of [4*R*-^{2}H] NADH (**A**) or NADH (**B**) with dye-linked L-proline dehydrogenase complex and Cl2Ind. *Arrow* shows the position of the C-4 proton of NAD

and Superdex 200 column chromatographies, respectively. The performance of purification at higher pHs seems to cause a large reduction in the specific activity of final preparation. The procedure for the recombinant enzyme purification does not need the use of such high pH buffer and is much more rapid than that used for the native enzyme purification. That may result in the higher specific activity of recombinant enzymes.

From the sequence database, the amino acid sequences of *T. profundus* enzyme α, γ, δ, and β subunits showed high homology with those deduced from the PAB0212, PAB0213, PAB3086, and PAB0214 genes of *P. abyssi* and those from PF1795, PF1796, PF1797, and PF1798 genes of *P. furiosus*. In addition, the structure of the *P. abyssi* gene cluster is found to be quite similar to that of the *T. profundus* one (Fig. 9), and the mutual neighboring genes are partially overlapped as in the case of *T. profundus*: the 3′ parts of PAB0212, PAB0213, and PAB3086 are overlapped with the 5′ parts of PAB0213, PAB3086, and PAB0214, respectively. The same characteristic was observed for the *P. furiosus* gene cluster (Fig. 9). Although the *pdh*X homologue of *P. horikoshii* and the *pdh*F and *pdh*X homologues of *A. fulgidus* were not identified in their databases, we found a *pdh*X homologue (PH*pdh*X) between PH1750 and PH1751 in the *P. horikoshii* gene cluster and *pdh*F and *pdh*X homologues (AF*pdh*F and AF*pdh*X, respectively) between AF0273 and AF0274 in the *A. fulgidus* one (Fig. 9). The amino acid sequence of AF*pdh*F exhibited a 52% homology with that of the γ subunit of the *T. profundus* enzyme, and the motif of the iron-sulfur cluster was well conserved in both protein sequences (Fig. 5). The amino

T. profundus	*pdh*A	*pdh*F	*pdh*X	*pdh*B
P. abyssi	PAB0212	PAB0213	PAB3086	PAB0214
P. horikoshii	PH1749	PH1750	(PH*pdh*X)	PH1751
P. furiosus	PF1795	PF1796	PF1797	PF1798
A. fulgidus	AF0273	(AF*pdh*F)	(AF*pdh*X)	AF0274

Fig. 9 Gene clusters of dye-linked L-proline dehydrogenase genes from *T. profundus* and similar gene clusters in *P. abyssi, P. horikoshii, P. furiosus, and A. fulgidus*. *Parenthesis* shows that the ORFs are identified in this study

acid sequences deduced from PH*pdh*X and AF*pdh*X exhibited 65% and 42% homologies, respectively, with that of the *T. profundus* δ subunit (Fig. 6). In the previous report, we described that dye-L-proDH activity is detected in several *Thermococcus* and *Pyrococcus* strains containing *P. horikoshii* and *P. furiosus* (Sakuraba et al. 2001). This suggests that similar dye-L-proDH complexes are distributed and play a characteristic physiological role in hyperthermophilic archaea belonging to the Thermococcales family.

In this study, the function of each of the four different subunits of the dye-L-proDH complexes was examined. We have succeeded in separate expression of four different genes in *E. coli* and found that the β subunit catalyzes the dehydrogenation of L-proline. To determine the function of the α subunit, we analyzed its primary structure in detail. Although the amino acid sequence showed high homologies with those of the putative sarcosine oxidase α subunit or D-nopaline dehydrogenase, the two activities were not detectable. On the other hand, the amino acid sequence of the α subunit exhibited lower homologies (about 30%) with those of NADH oxidases or NADH dehydrogenases from other microorganisms. Thus, we assayed the dye-NADHDH activity of the α subunit and found that the subunit itself has NADH dehydrogenase activity with Cl2Ind as an electron acceptor. The activity was specific for NADH, and NADPH was inert as the electron donor. The higher dye-NADHDH activity was also detected for the enzyme complex. The specific activities of the α and β subunits are much less than those of the enzyme complex, suggesting that the catalytic abilities of NADH and L-proline dehydrogenations are enhanced by the complex formation. In addition, we determined the stereospecificity of hydrogen transfer for NADH catalyzed with the NADH dehydrogenase by ^{1}H NMR resonance. In general, NADH- and NADPH-dependent dehydrogenases and reductases are well known to show pro-*R* or pro-*S* stereospecificity for hydrogen removal from the C-4 position of the reduced nicotine amide moiety by the ^{1}H NMR method. NADH dehydrogenase showed a pro-*S*-specific hydride transfer (B-type). Two different stereospecificities of hydrogen transfer for enzymes exhibiting NAD(P)H dehydrogenase activity from various sources have so far been reported (Hugh and Ian 1983): one is pro-*R*-specific enzymes such as NADH [cytochromeb_5 reductase (microsomal)], NADH dehydrogenase (mitochondrial and external), and NAD(P)H [quinone dehydrogenase (rat liver)]; and the other is pro-*S*-specific enzymes such as respiratory NADH dehydrogenase (*E. coli* and mitochondrial) and thioredoxin reductase. Thus, NADH dehydrogenase in the L-proline dehydrogenase complex belongs to the latter group. The difference in NAD(P)H-hydride transfer stereospecificity is considered to reflect the difference in formation of the nicotine amide binding domain in the molecular revolution of dehydrogenases and reductases, and large structural differences have been observed between pro-*S*- and pro-*R*-specific dehydrogenases such as the cases of leucine dehydrogenase (pro-*S* specific) and alanine dehydrogenase (pro-*R* specific) (Esaki et al. 1989). Our results on the stereospecificity of hydride transfer for the NADH dehydrogenase may afford some information on the bases of the structural formation of NAD(P)H-dependent enzymes because this is the first example of stereospecificity determination for hydride transfer of archaeal dehydrogenases and of NADH dehydrogenase in a dye-linked amino acid dehydrogenase complex.

On the primary structure, we have detected some specific motifs in the α, β, and γ subunits: the α subunit has one [2Fe-2S] motif and two ADP-binding motifs; the β subunit has one ADP-binding motif; and the γ subunit has two [4Fe-4S] motifs. Spectra of the α and β subunits suggest that these subunits contain flavin compounds. Thus, we determined the flavin compounds by HPLC and detected FAD from both subunits. About 2 mol of FAD per mol of the enzyme complex is detected, but only 0.1 and 0.2 mol of FAD per mol of α and β subunits, respectively, are detected. These results suggest that the enzyme complex contains 1 mol of FAD per mol of each α and β subunit but that the α and β subunits independently expressed can not adequately bind FAD. Both the α and γ subunits contain the iron-sulfur cluster motifs. Spectra of the α and γ subunits showed the typical [2Fe-2S] iron-sulfur flavoprotein and [8Fe-8S] ferredoxin, respectively. We found that 8 mol of Fe were contained in 1 mol of enzyme complex in this study. In the spectrum of the enzyme complex, a peak at 340 nm, which arose from the [2Fe-2S] cluster, was not observed (Fig. 7D). In addition, after the purification of the α subunit, the peak at 340 nm gradually disappeared. These results show that the [2Fe-2S] cluster in the enzyme complex

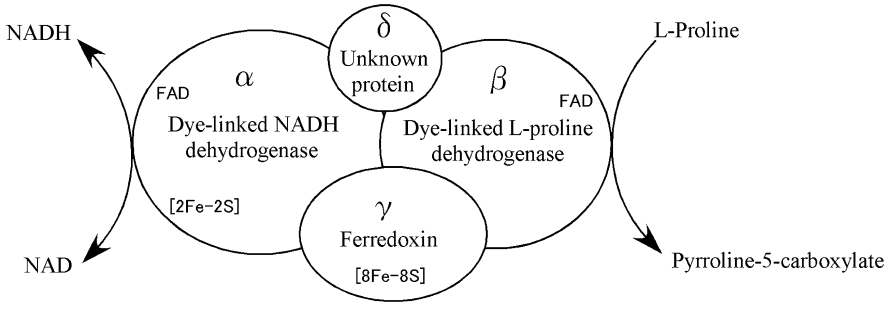

Fig. 10 Postulated scheme for the dye-linked L-proline dehydrogenase complex. Sakuraba et al. (2001) have already reported that the product of L-proline dehydrogenation was pyrroline-5-carboxylate

may be destroyed by air oxidation during the purification procedure.

In this study, we showed that the enzyme complex can catalyze two different reactions of dye-linked L-proline and NADH dehydrogenations and that it contains ferredoxin (Fig. 10). To our knowledge, this is the first example showing the existence of a bifunctional dye-linked L-proline/NADH dehydrogenase complex, and the functional and structural properties are totally different from the *E. coli* one. The *E. coli* enzyme is a homodimer with a subunit molecular weight of 144 kDa and it catalyzes both L-proline dehydrogenase and pyrroline-5-carboxylate dehydrogenase activities (Scarpulla and Soffer 1978).

In general, NADH dehydrogenase or dye-linked dehydrogenases are localized on the surface of the cytoplasmic membrane and play an important role in the incorporation of electrons from a substrate into the electron-transfer system. The enzyme complex contains the ferredoxin encoded by *pdh*F. The electrons may be transferred from L-proline to NAD via the protein. Details of the electron-transfer pathway from L-proline to NADH and the function of the δ subunit are still unknown.

The structural analysis of the dye-L-proDH complex and a further functional analysis of each subunit will provide abundant information on the relationship between the structure and function of each subunit.

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