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L-Threonine dehydrogenase from the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3: gene cloning and enzymatic characterization

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Abstract A gene encoding the L-threonine dehydrogenase homologue has been identified in a hyperthermophlic archaeon *Pyrococcus horikoshii* OT3 via genome sequencing. The gene was cloned and expressed in *Escherichia coli*. The purified enzyme from the recombinant *E. coli* was extremely thermostable; the activity was not lost after incubation at 100°C for 20 min. The enzyme (molecular mass: 192 kDa) is composed of a tetrameric structure with a type of subunit (41 kDa). The enzyme is specific for NAD and utilizes L-threonine, L-serine and DL-*threo*-3-phenylserine as the substrate. The enzyme required divalent cations such as Zn^{2+}, Mn^{2+} and Co^{2+} for the activity, and contained one zinc ion/subunit. The K_m values for L-threonine and NAD at 50°C were 0.20 mM and 0.024 mM, respectively. Kinetic analyses indicated that the L-threonine oxidation reaction proceeds via a random mechanism with regard to the binding of L-threonine and NAD. The enzyme showed pro-*R* stereospecificity for hydrogen transfer at the C4 position of the nicotinamide moiety of NADH. This is the first description of the characteristics of an L-threonine dehydrogenase from the archaea domain.

Keywords *Pyrococcus horikoshii* OT3 · L-Threonine dehydrogenase · Hyperthermophile · Archaea · The stereospecificity of the hydrogen transfer

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Introduction

L-Threonine dehydrogenase (L-ThrDH, EC 1.1.1.103) catalyzes the NAD-dependent oxidation of L-threonine. The suspected product of the reaction is 2-amino-3-oxobutyrate, which spontaneously decomposes to yield aminoacetone and CO_2 (Elliott 1958) or is further cleaved in a CoA-dependent reaction to produce glycine and acetyl-CoA (McGilvray and Morris 1969; Aoyama and Motokawa 1981). The L-ThrDH so far found catalyzes the dehydrogenation at the β-carbon (C3) position of L-threonine, while other amino acid dehydrogenases such as glutamate dehydrogenase and alanine dehydrogenase (Ohshima and Soda 2000) catalyze the dehydrogenation at the α-carbon (C2) position of the amino acid accompanying the deamination. In this respect, the enzyme is regarded as a kind of alcohol dehydrogenase. L-ThrDH is recognized to catalyze the initial reaction in the L-threonine degradation pathway, which is the primary route for threonine utilization in both eucaryotes and bacteria (Aoyama and Motokawa 1981; Boylan and Dekker 1981; McGilvray and Morris 1969; Epperly and Dekker 1991). L-ThrDH was purified to homogeneity from *Escherichia coli* K-12 (Boylan and Dekker 1981), a psychrophilic bacterium *Cytophaga* sp. KUC-1 (Kazuoka et al. 2003), *Clostridium sticklandii* (Wagner and Andreesen 1995), chicken liver mitochondria (Aoyama and Motokawa 1981), goat liver mitochondria (Ray and Ray 1985), and porcine liver mitochondria (Kao and Davis 1994). However, only the enzymes from *E. coli* and *Cytophaga* sp. have been intensively characterized, and the primary structures were determined (Aronson et al. 1989; Kazuoka et al. 2003). As yet, no L-ThrDH has been reported either in the archaea, the third domain of life, or in hyperthermophiles. We now present the first report of the biochemical characterization of an L-ThrDH from *Pyrococcus horikoshii* OT3, a hyperthermophilic archaeon that optimally grows at 98°C.

Materials and methods

Materials

The plasmid DNA pTrcHisA and *E. coli* stain TOP10 cells were purchased from Invitrogen (USA). The restriction enzymes were obtained from New England Biolabs (Tokyo, Japan). Deuterated L-serine (2,3,3-d_3) and ^2H_2O were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). All other chemicals were of reagent grade.

Cloning and expression of the gene encoding L-ThrDH

The complete sequence of the *P. horikoshii* OT3 genome has been reported by Kawarabayasi et al. (1998). The gene, which is homologous to that of the *E. coli* L-ThrDH, was found by using the BLAST server in the GenBank databases. The plasmid DNA (p1962: position 587065–589454 on the entire genome of *P. horikoshii* OT3 has been inserted into the *Hin*cII site of pUC 118) containing an open reading frame of the gene (ORF ID: PH0655, position 587615–588661 on the entire genome) was prepared from the fosmid clone as previously described (Kawarabayasi et al. 1998). The following set of two oligonucleotide primers were used for amplification of the PH0655 gene by PCR. The forward primer of 5′-GATGGATCCATGTCAGAAAAGATGG TAG-3′ was designed on the basis of the N-terminal amino acid sequence of *P. horikoshii* L-ThrDH, and a unique *Bam*HI restriction site was introduced. The reverse primer of 5′-GTAAAGCTTTC ATTTAAGCATAAAA ACA-AC-3′ was designed on the basis of the C-terminal amino acid sequence and a unique *Hin*dIII restriction site was introduced. The plasmid DNA p1962 was used as the template. The amplified 1 kb fragment was digested with *Bam*HI and *Hin*dIII, and ligated into the expression vector pTrcHisA linearized with *Bam*HI and *Hin*dIII to generate pThrDH. The *E. coli* TOP10 cells were transformed with pThrDH, and the transformants were cultivated at 37°C for 10 h in a Luria-Bertani medium (2 L) containing 50 μg/ml of ampicillin.

Purification of recombinant L-ThrDH

Cells were harvested by centrifugation and suspended in 10 mM MOPS/NaOH buffer (pH 7.0) containing 5 mM 2-mercaptoethanol (2-ME), 1 mM EDTA and 0.1 mM benzylsulfonyl fluoride. The cells were disrupted by ultrasonication and then centrifuged (15,000× g for 10 min). The supernatant (the crude extract) was heated at 85°C for 10 min and the denatured protein was removed by centrifugation (15,000× g for 10 min). The supernatant was dialyzed against 10 mM MOPS/NaOH buffer (pH 7.0) containing 5 mM 2-ME and 0.1 mM $ZnCl_2$. The solution was loaded on a Ni^{2+}-chelating Sepharose column (1.5 cm × 3 cm, Chelating Sepharose Fast Flow, Amersham Biosciences, USA) equilibrated with 10 mM MOPS/NaOH buffer (pH 7.0) containing 5 mM 2-ME, 0.1 mM $ZnCl_2$, and 10 mM imidazole. After washing with the same buffer, the enzyme was eluted with a 50-ml linear gradient of 10 mM–2 M imidazole in the same buffer. The active fractions were pooled, dialyzed against 10 mM MOPS/NaOH buffer (pH 7.0) containing 5 mM 2-ME and 0.1 mM $ZnCl_2$, and used as the purified enzyme preparation.

Zn^{2+} content

The enzyme was purified with the above method, which used the same buffer except $ZnCl_2$. The purified enzyme was concentrated by Centrifugal Filter (Amicon Ultra PL-30, Millipore, MA, USA) and the filtrate was used as a blank. The Zn^{2+} content of the enzyme (1 mg/ml) was analyzed using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICPS-8000, Shimadzu, Kyoto, Japan).

Enzyme assay and protein determination

The L-ThrDH activity was spectrophotometrically assayed by using a Shimadzu UV-160A spectrophotometer equipped with a thermostat. The standard reaction mixture was composed of 100 mM glycine/NaOH buffer (pH 10.0), 2 mM L-threonine, 0.4 mM NAD, 0.05 mM $ZnCl_2$ and enzyme in a final volume of 1.00 ml. The reaction mixture was incubated at 50°C for 5 min, and the change in the NADH absorbance was monitored at 340 nm ($\epsilon = 6.2\ mM^{-1}\ cm^{-1}$). One unit of enzyme is defined as the amount catalyzing the formation of 1 μmol NADH per min at 50°C in the L-threonine oxidation. The protein concentration was determined by the method of Bradford using the Bio-Rad Protein Assay Kit (Bradford 1976). Bovine serum albumin was used as the standard.

Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (PAGE, 12.5% acrylamide slab gel, 1 mm thick) was performed using the procedure of Laemmli (Laemmli 1970). The protein band was stained with Coomassie Brilliant Blue R 250.

Molecular Mass Determination

The molecular mass of the purified enzyme was determined by an analytical gel filtration on a Superose 6 HR 10/30 column (1 × 30 cm; Amersham Biosciences) equilibrated with 10 mM MOPS/NaOH buffer (pH 7.0) containing 5 mM 2-ME, 0.1 mM $ZnCl_2$ and 0.2 M NaCl. Gel Filtration Calibration Kits (Amersham Biosciences) and glutamate dehydrogenase from *Pyrobacu-*

lum islandicum (Kujo et al. 1999) were used as the molecular mass standards. The subunit molecular mass of the purified enzyme was determined by SDS-PAGE using six marker proteins (New England BioLabs).

N-terminal amino acid sequencing

After SDS-PAGE of the purified L-ThrDH, the protein was electrophoretically transferred onto a poly (vinylidene fluoride) membrane (Bio-Rad, Japan) and stained with Ponceau S. The stained protein band was cut out and the N-terminal amino acid sequence of the protein was determined using the protein sequencer PPSQ-10 (Shimadzu).

Stability, and temperature and pH optima

To determine the pH stability, the enzyme in buffers of various pH values was incubated at 50°C for 20 min, and the remaining activities were then assayed. The buffers (100 mM) were an acetate buffer, MOPS/NaOH buffer, glycylglycine/NaOH buffer, glycine/NaOH buffer and sodium phosphate/NaOH buffer in the pH ranges of 3.5–6.5, 6.5–8.0, 8.0–9.0, 9.0–10.5 and 10.5–12.0, respectively. The optimal pH of the enzyme reaction was determined by running the standard assay at 50°C using the glycylglycine/NaOH buffer (200 mM), glycine/NaOH buffer (200 mM) and sodium phosphate/NaOH buffer (200 mM) in the pH ranges of 8.0–9.0, 9.0–11.0 and 11.0–12.0, respectively. To determine the thermostability, the enzyme solutions in 10 mM MOPS/NaOH buffer (pH 7.0) were incubated at different temperatures, and the residual activity was determined by the standard assay method. The optimal temperature of the reaction was determined by running the standard assay at temperatures from 45°C to 80°C. The effects of the organic solvents on the enzyme stability were examined by measuring the activity remaining after incubation (50°C for 20 min) with various concentrations of the reagents. The organic solvents used were dimethyl sulfoxide (DMSO), ethanol and methanol.

Substrate specificity, metal ion requirement and kinetic analysis

The reactivities of L-threonine, L-*allo*-threonine, D-threonine, D-*allo*-threonine, L-alanine, L-valine, L-leucine, L-isoleucine, L-methionine, L-serine, L-proline, L-phenylalanine, L-asparagine, L-glutamine, L-cystein, L-lysine, L-histidine, L-aspartate, L-glutamate, glycine, D-serine, DL-*threo*-3-hydroxynorvaline, DL-*threo*-3-phenylserine, L-norvaline, L-homoserine, L-2-aminobutyrate, DL-homoserine, 2-amino butanol and DL-2-hydroxybutyrate acid as substrates for L-ThrDH were examined. The reaction mixture was composed of 200 mM glycine/NaOH (pH10.0), 3 mM of each substrate, 0.5 mM NAD, 0.1 mM $ZnCl_2$ and enzyme in a final volume of 1.00 ml. To determine the metal ion requirement, the enzyme solution was dialyzed against 10 mM MOPS/NaOH buffer (pH 7.0) containing 5 mM 2-ME and 1 mM EDTA, followed by a dialysis against the same buffer solution without EDTA. The reactivity of the divalent cation was tested by the addition of 0.1 mM $MgCl_2$, $CaCl_2$, $ZnCl_2$, $NiCl_2$, $CoCl_2$, $CuCl_2$, $CdCl_2$ or $MnCl_2$ to the reaction mixture. The steady-state kinetic mechanism study was carried out by initial velocity and product inhibition analyses (Cleland 1979).

Stereochemical analysis of hydrogen transfer for NADH

The stereospecificity of the hydrogen transfer for NADH was analyzed by ^1H NMR. The reaction mixture contained *P. horikoshii* L-ThrDH (1 U), 60 mM non-deuterated or deuterated L-serine, 1 mg NAD, 0.05 mM $ZnCl_2$ and 0.5 M NH_4HCO_3 buffer (pH 7.8) in a final volume of 1 ml. After incubation at 50°C for 2 h, the solution was passed through a Centrifugal Filter (Amicon Ultra PL-30). The filtrate was further purified by a DEAE sepharose column according to the method of Arnold et al. (1976). The samples were lyophilized twice from 99.8% ^2H_2O and dissolved in 99.9% ^2H_2O (Arnold et al. 1976). The ^1H NMR spectra of the C4 position on the nicotinamide ring of NADH were recorded on a 400 MHz ^1H NMR instrument (JEOL, Japan).

Results and discussion

Expression of the gene and purification of the recombinant enzyme

A gene PH0655 (1,047 bp), which may encode a putative L-ThrDH (a protein of 348 amino acids with a molecular weight of 37,785), has been identified in *P. horikoshii* via genome sequencing. We performed the cloning and expression of the PH0655 gene in *E. coli* TOP 10. The produced enzyme was purified about 7.3-fold with a 38% recovery by heat treatment and a Ni^{2+} -chelating affinity chromatography. About 17.6 mg of the purified enzyme was obtained from 2 L of the *E. coli* culture. The specific activity of the final preparation was estimated to be 3.43 U/mg (Table 1). The purified enzyme showed a single protein band by SDS-PAGE (Fig. 1a). The N-terminal amino acid sequence of the purified enzyme corresponded to the predicted amino acid sequence including the histidine-tag in N-terminal domain.

Molecular and catalytic properties

The molecular mass of a subunit was determined to be about 41.2 kDa by SDS-PAGE (Fig. 1a). This value is in fair agreement with the molecular weight (41,815) calculated from the amino acid sequence including the histidine-tag sequence. The molecular mass of L-ThrDH

Table 1 Purification of the recombinant L-ThrDH

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Crude extract	340	158	0.465	100
Heat treatment	75.8	198	2.61	125
Ni^{2+} affinity column	17.5	60.6	3.43	38.3

was estimated to be about 192 kDa by gel filtration. This suggests that the native L-ThrDH is a homotetrameric structure. Multiple oligomeric structures, such as the monomeric, dimeric and tetrameric forms have been observed for the L-ThrDHs from bacteria and eukarya. The enzymes from chicken liver (Aoyama and Motokawa 1981) and goat liver (Ray and Ray 1985) have a monomeric structure. The L-ThrDHs from *C. sticklandii* (Wagner and Andreesen 1995) and porcine liver (Kao and Davis 1994) have been reported to form a dimeric structure. The *E. coli* L-ThrDH forms a tetrameric structure (Boylan and Dekker 1981). In this regard, the *P. horikoshii* L-ThrDH is similar to the *E. coli* enzyme.

The effect of pH on activity was examined and the highest activity was observed around pH 10. Below and above pH 10, the marked decrease of activity was observed: the relative activities were 50, 22 and 55% at pH 9.5, 9.2 and 12, respectively. The enzyme exhibited a maximum activity at 70°C based on the temperature profile at pH 10. The enzyme was highly thermostable; it retained more than 90% activity up to 100°C by incubation for 20 min at pH 7.0. The activity was retained upon heating at 90°C for 30 min and at 80°C at least for 60 min. The remarkable loss of activity was observed above 105°C and the complete inactivation occurred upon 120°C for 20 min using an autoclave. Up to now, the thermostability of L-ThrDH has been reported only for the enzymes from *Staphylococcus aureus* (Green and Elliott 1964) and *Cytophaga* sp. (Kazuoka et al. 2003). Both enzymes are rapidly inactivated above 50°C. The presence of L-ThrDH has not yet been described in thermophilic organisms including hyperthermophiles. Thus, the *P. horikoshii* enzyme is probably the most thermostable L-ThrDH among the enzymes described to date. The enzyme was stable over a wide pH range; upon heating at 50°C for 20 min, the enzyme did not lose activity at pH 4.5–10.0. The enzyme was fairly resistant to organic solvents such as ethanol, methanol and DMSO at 50°C. It retains about 80% activity in the presence of these reagents even at a concentration as high as 30% (data not shown).

The substrate specificity of *P. horikoshii* L-ThrDH was examined. The enzyme was specific for the L-isomer of threonine, while three other isomers, L-*allo*-threonine, D-threonine and D-*allo*-threonine were inert. The enzyme also catalyzed the oxidation of L-serine (relative activity to that of L-threonine: 21%) and DL-*threo*-3-phenylserine (53%), but many other L-amino acids containing L-alanine, L-aspartate, L-valine, L-leucine, L-isoleucine, L-methionine, L-proline, L-phenylalanine, L-asparagine, L-glutamine, L-cystein, L-lysine, L-histidine, L-glutamate, DL-homoserine, 2-amino butanol, DL-2-hydroxybutyrate and glycine were inert. This suggests that the β-methyl group of L-threonine is not essential for the enzyme reaction and can be replaced by a phenyl group. In this regard, the *P. horikoshii* enzyme is similar to the *E. coli* one (Boylan and Dekker 1981). On the other hand, the *E. coli* L-ThrDH acts on DL-*allo*-threonine, but not on the *P. horikoshii* enzyme, showing that the *P. horikoshii* enzyme recognizes the stereostructure of a substrate

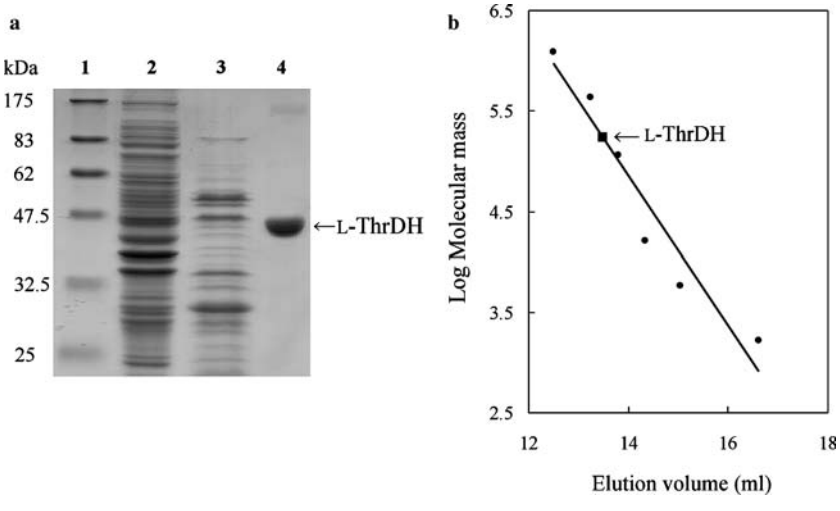

Fig. 1 SDS-PAGE of the purified recombinant L-ThrDH (**a**): *lane 1* the marker proteins, *lane 2* crude extract, *lane 3* heat treatment and *lane 4* purified L-ThrDH after Ni^{+} affinity column. The marker protein used are fusion of *Escherichia coli* maltose-binding protein and β-galactosidase (175 kDa), fusion of *E. coli* maltose-binding protein and paramysin (83 kDa), bovine glutamate dehydrogenase (62 kDa), rabbit muscle aldolase (47.5 kDa), rabbit muscle triosephosphate isomerase (32.5 kDa). A calibration curve for the determination of native molecular mass (**b**). The marker proteins used for gel filtration are horse spleen ferritin (440 kDa), *Pyrobaculum islandicum* glutamate dehydrogenase (282 kDa), rabbit muscle aldolase (158 kDa), bovine serum albumin (67 kDa), hen egg ovalbumin (43 kDa) and bovine pancreas chymotrypsinogen (25 kDa)

more strictly than the *E. coli* enzyme, as well as that described for the *Cytophaga* enzyme (Kazuoka et al. 2003). The *P. horikoshii* L-ThrDH did not utilize NADP as a cofactor similar to the enzymes from other sources.

The *P. horikoshii* L-ThrDH required Zn^{2+} for the activity similar to the *E. coli* enzyme (Boylan and Dekker 1981). When the *P. horikoshii* enzyme was dialyzed against a buffer containing 1 mM EDTA, the enzyme activity completely disappeared. After removal of EDTA, the full activity was recovered by the addition of 0.1 mM $ZnCl_2$. $ZnCl_2$ was able to be replaced by $MnCl_2$ (relative activity to that of Zn^{2+} : 77%) or $CoCl_2$ (35%) to some extent. The *E. coli* enzyme is known to still retain about 70% of the original activity even after dialysis against 0.1 M EDTA (Boylan and Dekker 1981). This indicates that the binding of Zn^{2+} to the *P. horikoshii* enzyme is much weaker than that to the *E. coli* enzyme. From the analysis of Zn^{2+} content of the enzyme by Inductively Coupled Plasma Atomic Emission Spectroscopy, the enzyme contains 0.89 g-atom of zinc per subunit. This result shows that the enzyme has one zinc atom per subunit. The value is similar to *E. coli* enzyme (Epperly and Dekker 1991).

The steady-state kinetic mechanism for the L-ThrDH reaction was investigated to identify the reaction mechanism. Double-reciprocal plots of the initial velocity versus the L-threonine concentrations in the presence of several fixed concentrations of NAD gave intersecting straight lines (Fig. 2a). This pattern shows that the reaction proceeds via the formation of a ternary complex of the enzyme with L-threonine and NAD. The K_m values for L-threonine and NAD were determined to be 0.20 and 0.024 mM, respectively, from replots of the reciprocal fixed substrate concentrations and vertical intersects.

Product inhibition studies for the oxidation of L-threonine were possible only with NADH, because 2-amino-2-oxobutyrate is unstable (Misono et al. 1987). With NADH as an inhibitor, the double-reciprocal plots of the initial velocity versus the NAD concentrations at a constant concentration of L-threonine showed a typical competitive inhibition pattern (Fig. 2b). NADH also showed a similar competitive inhibition pattern against L-threonine (Fig. 2c). These inhibition patterns support the concept that NAD and L-threonine randomly bind to the enzyme, and NADH is released from the enzyme as the last product, but it is still unclear as to the sequential ordered release of 2-amino-3-oxobutyrate and NADH from where the enzyme occurs. The enzyme reaction of the L-ThrDHs from *Cytophaga* and chicken liver has been reported to proceed via an ordered Bi-Bi mechanism in which NAD binds to the enzyme prior to

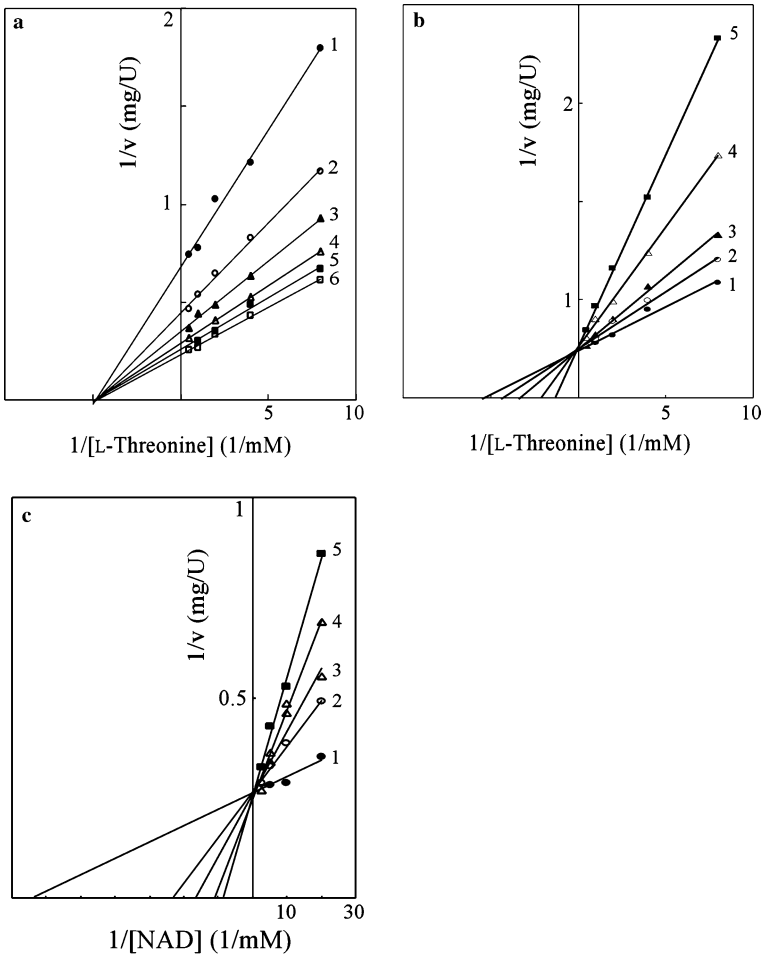

Fig. 2 **a** Double reciprocal plots of initial velocity against L-threonine concentration at a series of fixed concentrations of NAD. The concentration of NAD were 1, 0.0125; 2, 0.025; 3, 0.05; 4, 0.1; 5, 0.2 and 6, 0.4 mM. **b, c** Product inhibition patterns of NADH with the varied substrate concentrations of NAD (**b**) and L-threonine (**c**). The concentrations of NADH used were 1, 0; 2, 0.015; 3, 0.03; 4, 0.04 and 5, 0.06 mM in (**b**) and 1, 0; 2, 0.015; 3, 0.03; 4, 0.06 and 5, 0.1 mM in (**c**)

L-threonine (Kazuoka et al. 2003; Aoyama and Motokawa 1981). Thus, the *P. horikoshii* L-ThrDH may be unique in the reaction mechanism among the L-ThrDHs described to date.

Stereospecificity of hydrogen transfer from L-serine to NADH

In general, a deuterium (hydride) of the deuterium-labeled substrate is stereospecifically transferred to the C4 position of the pyridine ring in the NAD molecule by a dehydrogenase reaction, and *R* or *S*-stereospecifically dueterated NADH is produced. Since the *P. horikoshii* L-ThrDH catalyzes the dehydrogenation of L-serine in the presence of NAD, we examine the stereospecificity of the hydrogen transfer of the L-ThrDH reaction using the deuterium labeled L-serine. The hydrogen configuration at the C4 position in the pyridine ring of NADH formed by the enzymatic reaction was examined using the ^{1}H NMR spectrum. The ^{1}H NMR spectrum of the proton at C4 in the pyridine ring of NADH shows two resonance peaks, which are both split by the geminal coupling between the two C4 protons (Mostad et al. 1997). These resonances were observed at 2.50, 2.55, 2.62 and 2.67 ppm in the 400 MHz ^{1}H NMR spectrum (Fig. 3a, the spectral region between 2.4 and 2.7 ppm is shown). When unlabeled L-serine was incubated with the enzyme in the presence of NAD, similar resonance peaks of NADH were observed in the ^{1}H NMR spectrum (Fig. 3b). Figure 3c shows the ^{1}H NMR spectrum of NADH formed when deuterated L-serine (2,3,3-d_3) was used as the substrate. The spectrum peaks showed a symmetrical chemical shift due to the 4 *S* proton–pyridine proton 5 coupling (Mostad et al. 1997), indicating the formation of [4 *R*–^{2}H, 4 *S*–^{1}H] NADH from the deuterated L-serine (2,3,3-d_3) and NAD by the enzyme reaction. These results indicate that the *P. horikoshii* L-ThrDH exhibits a pro-*R*-specificity for the hydrogen transfer (A-type stereospecificity). This is the first example of the stereospecific determination for the hydrogen transfer of L-ThrDH. L-ThrDH catalyzes the β-dehydrogenation of L-threonine and L-serine, and belongs to the family of alcohol dehydrogenase. Schneider-Bernlöhr et al. (1986) postulated that the zinc-containing alcohol dehydrogenase family with a larger subunit molecular mass (33–42 kDa) exhibits a pro-*R*-specific hydrogen transfer, and the family of zinc-noncontaining enzymes with a smaller subunit molecular mass (24–28 kDa) exhibits the pro-*S*-specific one. Our observation is in line with their hypothesis, because the *P. horikoshii* L-ThrDH requires Zn^{2+} for the activity and consists of the larger subunit molecular mass of 41 kDa.

Amino acid sequence analysis

The primary structure of L-ThrDH has been reported for the enzymes from *E. coli* (Aronson et al. 1989) and *Cytophaga* (Kazuoka et al. 2003). The amino acid sequence of *P. horikoshii* L-ThrDH was aligned with those of *E. coli* L-ThrDH, and horse liver alcohol dehydrogenase (isozyme E) (Fig. 4). The *P. horikoshii* enzyme exhibited a high sequence homology (43%) with the *E. coli* L-ThrDH. The *E. coli* enzyme shares a 25% amino acid identity with horse liver alcohol dehydrogenase, whose crystal structure has already been solved (Eklund et al. 1981, 1984). The important amino acid residues reported for the horse liver alcohol dehydrogenase [i.e. Cys-46, Cys-97, Cys-100, Cys-103, Cys-111 and His 67 (residue numbers of the horse liver alcohol dehydrogenase were given)] are strictly conserved in the *E. coli* L-ThrDH (Aronson et al. 1989), and both enzymes are classified into the medium-chain alcohol dehydrogenase family. These residues are highly conserved in the

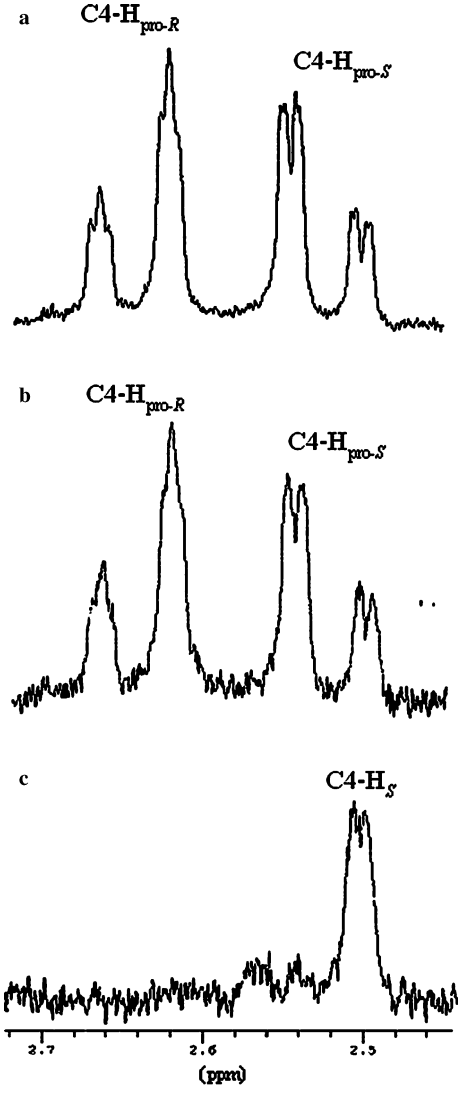

Fig. 3 ^{1}H NMR spectra of the C4 hydrogens of NADH: standard unlabeled NADH (**a**), NADH formed by L-ThrDH reaction using nondeuterated L-serine and NAD (**b**), and NADH formed by the enzyme reaction using deuterated L-serine (2,3,3-d_3) and NAD (**c**)

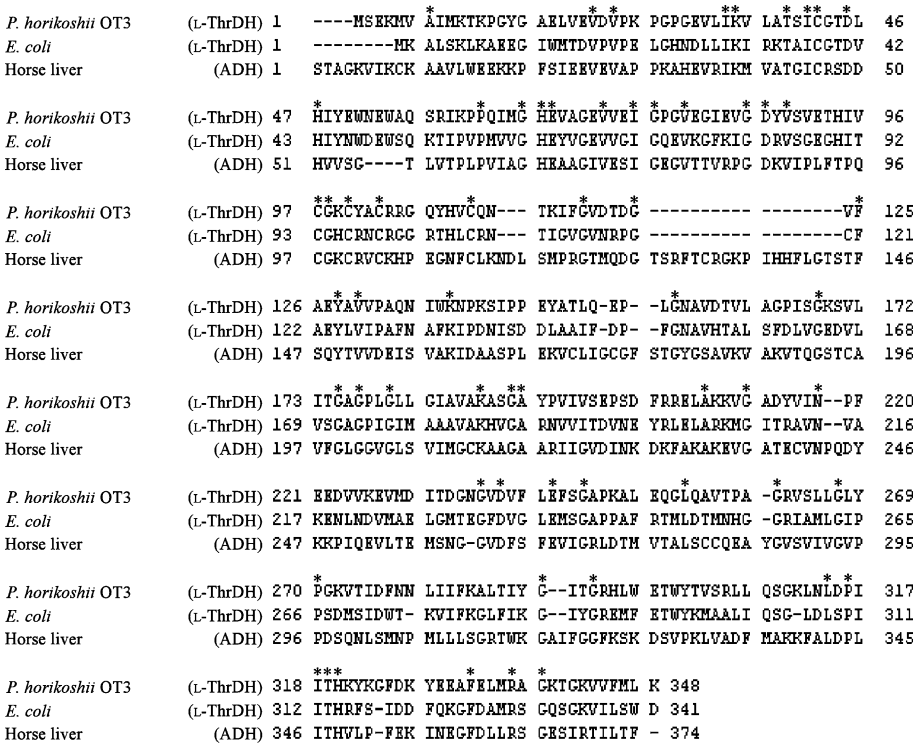

Fig. 4 Amino acid sequence comparison of *P. horikoshii* L-ThrDH with *E. coli* L-ThrDH and horse liver alcohol dehydrogenase isozyme E (ADH). The proposed alignment was constructed with the multiple alignment program in ClustalW (Thompson et al. 1994). *Asterisks* represent the conserved residues in the three enzymes

sequence of *P. horikoshii* L-ThrDH (Fig. 4), suggesting that the enzyme also belongs to the same family. The homologues of the *P. horikoshii* enzyme appear to be present in several hyperthermophilic archaea whose entire genomes have been sequenced. For example, *P. horikoshii* L-ThrDH exhibited 93% and 90% identities with probable L-ThrDHs of *P. abyssi* (ORF PAB2382) and *P. furiosus* (PF0991), respectively. On the other hand, *Cytophaga* L-ThrDH has been reported to show no sequence similarity with *E. coli* L-ThrDH (Kazuoka et al. 2003). Interestingly, the *Cytophaga* enzyme exhibits a significant sequence identity with UDP-glucose 4-epimerases from the mesophilic bacterion *Staphylococcus aureus* (50%) and the thermophilic archaeon *Thermoplasma volcanium* (43%) (Kazuoka et al. 2003). These enzymes belong to the short-chain dehydrogenases-reductase superfamily unlike medium-chain alcohol dehydrogenases. This suggests that two different types of L-ThrDH occur in archaea as well as in bacteria. The hyperthermophilic archaea are relatively deeply branched archaea and are considered to be phylogenetically ancient organisms. Therefore, it is interesting to examine the distribution of both types of the L-ThrDHs in the hyperthermophilic archaea. This may offer some important information on the evolutionary relationship and physiological role of L-ThrDHs in microorganisms.

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