Extremophiles (2002) 6:275–281
Digital Object Identifier (DOI) 10.1007/s00792-001-0254-3

ORIGINAL PAPER

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L-Aspartate oxidase is present in the anaerobic hyperthermophilic archaeon *Pyrococcus horikoshii* OT-3: characteristics and role in the de novo biosynthesis of nicotinamide adenine dinucleotide proposed by genome sequencing

Received: June 1, 2001 / Accepted: October 12, 2001 / Published online: March 8, 2002

Abstract A gene encoding the L-aspartate oxidase homologue was identified via genome sequencing in the anaerobic hyperthermophilic archaeon *Pyrococcus horikoshii* OT-3. We succeeded in expressing the encoding gene in *Escherichia coli* and purified the product to homogeneity. Characterization of the protein revealed that it is the most thermostable L-aspartate oxidase detected so far. In addition to the oxidase activity, the enzyme catalyzed L-aspartate dehydrogenation in the presence of an artificial electron acceptor such as phenazine methosulfate, 2,6-dichlorophenol-indophenol, and ferricyanide. L-Aspartate oxidase is known to function as the first enzyme in the de novo NAD biosynthetic pathway in prokaryotes. By a similarity search in public databases, the genes that encode the homologue of all other enzymes involved in the pathway were identified in the *P. horikoshii* OT-3 genome. This suggests that *P. horikoshii* OT-3 may use the de novo NAD biosynthetic pathway under anaerobic conditions.

Key words L-Aspartate oxidase · *Pyrococcus horikoshii* OT-3 · NAD biosynthesis · Archaea · Hyperthermophile

Introduction

In prokaryotes, the de novo biosynthesis of NAD generally proceeds through the condensation reaction between L-aspartate and dihydroxyacetone phosphate, and the reaction is catalyzed by the quinolinate synthase system, a complex composed of two enzymes, L-aspartate oxidase and quinolinate synthase (Nasu et al. 1982). L-Aspartate oxidase catalyzes the oxidation of L-aspartate to iminoaspartate, using oxygen as an electron acceptor and producing hydrogen peroxide. The imino acid is then condensed with dihydroxyacetone phosphate to produce quinolinate by quinolinate synthase. Quinolinate is subsequently converted to NAD via a metabolic sequence common to all organisms (Magni et al. 1999). In spite of the physiological importance of the process, information about the characteristics of the L-aspartate oxidase that catalyzes the first reaction of the process is limited. The gene encoding L-aspartate oxidase has been cloned and sequenced from at least three prokaryotic organisms: *Escherichia coli*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* (Flachmann et al. 1988; Sun and Setlow 1993; DeVries et al. 1995). However, only the *E. coli* gene has been expressed and its product characterized (Seifert et al. 1990; Mortarino et al. 1996; Tedeschi et al. 1996; Mattevi et al. 1999).

As yet, no L-aspartate oxidase has been reported either in the Archaea, the third domain of life (Woese et al. 1990), or in obligate anaerobic organisms. We now present the first report of the biochemical characterization of an L-aspartate oxidase from *Pyrococcus horikoshii* OT-3, an anaerobic hyperthermophilic archaeon that grows optimally at 98°C (Gonzalez et al. 1998). In addition, we propose a role for the enzyme in de novo NAD biosynthesis on the basis of genome sequencing of *P. horikoshii* OT-3.

Communicated by K. Horikoshi

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Materials and methods

Materials

The *E. coli* strain BL21-CodonPlus-RIL was obtained from Stratagene (La Jolla, CA, USA). Horseradish peroxidase was obtained from Toyobo (Osaka, Japan). *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine was purchased from

Sigma (St. Louis, MO, USA). 4-Aminoantipyrine, β-NADH, and 2,6-dichlorophenolindophenol (DCIP) were purchased from Wako (Osaka, Japan). Malate dehydrogenase from yeast and lactate dehydrogenase from pig heart were obtained from Oriental Yeast (Tokyo, Japan). All other chemicals were of reagent grade.

Assay for L-aspartate oxidase activity

The standard assay for L-aspartate oxidase activity was carried out using the horseradish peroxidase-coupled method as previously described (Mortarino et al. 1996). The reaction system contained 50 mM Tris/HCl, pH 8.6, 0.66 mM *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine, 80 mM L-aspartate, 4.8 U/ml horseradish peroxidase, 0.48 mM 4-aminoantipyrine, 0.03 μM FAD, and enzyme in a total volume of 1.0 ml. The reaction was started by the addition of L-aspartate at 37°C and was monitored by using a Shimadzu UV-160A recording spectrophotometer (Kyoto, Japan) to follow the appearance of the quinoneimine dye at 550 nm. A unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of one micromole of hydrogen peroxide (half a micromole of quinoneimine dye) per minute at 37°C. The protein concentration was determined by the Bradford method (Bradford 1976).

Assay for L-aspartate/fumarate oxidoreductase activity

Iminoaspartate formed from L-aspartate by the reaction of the enzyme spontaneously hydrolyzes to yield oxaloacetate. Oxaloacetate was quantified by a malate dehydrogenase-coupled assay as previously described (Tedeschi et al. 1996). The reaction mixture contained 50 mM Tris/HCl, pH 8.6, 10 mM fumarate, 80 mM L-aspartate, 10 U/ml malate dehydrogenase, 10 U/ml lactate dehydrogenase, 0.03 μM FAD, 0.13 mM NADH, 20% glycerol, and enzyme in a total volume of 1.0 ml. The reaction was carried out at 37°C under anaerobic conditions. Anaerobiosis was achieved in a rubber-capped quartz cuvette by bubbling nitrogen gas for 3 min into the reaction mixture without L-aspartate, and the reaction was started by the addition of L-aspartate that had been kept under anaerobiosis by bubbling nitrogen gas. The activity was determined by following the decrease in the absorbance at 340 nm due to the oxidation of NADH.

Assay for dye-linked L-aspartate dehydrogenase activity

The standard reaction mixture was composed of 50 mM Tris/HCl, pH 8.6, 80 mM L-aspartate, 0.1 mM DCIP, 0.03 mM FAD, and enzyme in a total volume of 1.0 ml. The reaction was carried out at 37°C under anaerobic conditions in a cuvette with a 0.4-cm light path. Anaerobiosis was achieved by a method similar to that already described. The reaction was started by the addition of L-aspartate and followed by measuring the initial decrease in absorbance at 600 nm accompanying the reduction of DCIP. An ε_{mM} of 21.5 mM^{-1} cm^{-1} was used for the DCIP. The reduction of ferricyanide and *p*-iodonitrotetrazolium violet (INT)/phenazine methosulfate (PMS) was assayed at 405 nm (ε_{mM}=1.04 mM^{-1} cm^{-1}) and 490 nm (ε_{mM}=15.0), respectively.

Cloning of the gene

The complete sequence of the genome of *P. horikoshii* OT-3 has been reported by Kawarabayasi et al. (1998). The gene, which is homologous to that of the *E. coli* L-aspartate oxidase, was found by a BLAST search (Altschul et al. 1990). The plasmid DNA (p2496: position 12,152–14,467 on the entire genome of *P. horikoshii* OT-3, which was inserted into the *Hin*cII site of pUC118) containing an open reading frame of the gene (ORF ID: PH0015, position 12,928–14,323 on the entire genome) was prepared from the fosmid clone as previously described (Kawarabayasi et al. 1998).

Overexpression and purification of the recombinant protein

The *E. coli* strain BL21-CodonPlus-RIL was transformed with p2496. The transformants were cultivated at 37°C in 7 l of the medium containing bacto tryptone (12 g/l), yeast extract (24 g/l), glycerol (5 ml/l), K_2HPO_4 (12.5 g/l), KH_2PO_4 (3.8 g/l), and ampicillin (50 μg/ml) until the optical density at 600 nm reached 0.6. The induction was carried out by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside to the medium, and cultivation was continued for 3 h. Cells were harvested by centrifugation and suspended in 50 mM potassium phosphate buffer (pH 7.5). After incubation at 37°C for 30 min with stirring in the presence of lysozyme (1 mg/ml) and DNase (0.1 mg/ml), the cells were disrupted by ultrasonication. After centrifugation (15,000 *g* for 10 min), the soluble fraction of the extract was heated at 80°C for 30 min. The denatured protein was then removed by centrifugation (15,000 *g* for 10 min). The supernatant solution was brought up to 30% saturation of $(NH_4)_2SO_4$ and left at 4°C for 16 h with stirring. The precipitate was removed by centrifugation at 15,000 *g* for 30 min and discarded. $(NH_4)_2SO_4$ was added to the supernatant solution to 60% saturation, and the solution was left at 4°C for 6 h. The resulting precipitate was collected by centrifugation at 15,000 *g* for 30 min and dissolved in 50 mM potassium phosphate buffer (pH 7.5). The enzyme solution was desalted by dialysis against the same buffer. The enzyme solution was loaded on a Q-Sepharose fast-flow column (2.6 × 10 cm; Pharmacia, Tokyo, Japan) equilibrated with 50 mM potassium phosphate buffer (pH 7.5). After washing with 200 ml of the same buffer, protein was eluted with a 600-ml linear gradient of 0–1.0 M NaCl in the same buffer. The active fractions were pooled, concentrated by ultrafiltration, and dialyzed against 20 mM potassium phosphate buffer (pH 7.5), and then applied to a column (2 × 10 cm) of hydroxyapatite (GIGAPITE K-100S, Seikagaku Kogyo, Tokyo, Japan) equilibrated with the same buffer. After washing with 300 ml of the same buffer, the enzyme was

eluted with a 300-ml linear gradient of 20–500 mM potassium phosphate buffer, pH 7.5. The active fractions were pooled, dialyzed against 50 mM potassium phosphate buffer (pH 7.5), and used as the purified enzyme preparation.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE; 7.5% acrylamide gel) was carried out according to the method of Davis (1975), and SDS-PAGE (12% acrylamide slab gel, 1 mm thick) was performed using the procedure of Laemmli (1970).

Molecular mass determinations

The molecular mass of the native enzyme was measured using high performance liquid chromatography (HPLC) (Tosoh CCPE, Tokyo, Japan) with a gel filtration column (TSK gel column G3000SWXL, 7.8 mm × 30 cm, Tosoh). The following marker proteins (Bio-Rad, Hercules, CA, USA) were used to create a calibration curve: bovine thyroglobulin (molecular mass, 670 kDa), bovine β-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa). The subunit molecular mass of the enzyme was determined by SDS-PAGE.

N-terminal amino acid sequencing

Approximately 2.5 μg of purified L-aspartate oxidase was subjected to SDS-PAGE as already described, followed by electroblotting onto a polyvinylidene difluoride membrane. The membrane was then stained with Ponceau S and destained. A protein band was excised and subjected to automated Edman degradation using the Shimadzu Model PPSQ-10 protein sequencer.

Temperature and pH optima, thermal stability, and kinetic parameters

The optimal temperature was determined by assaying the dye-linked L-aspartate dehydrogenase with ferricyanide as an electron acceptor at temperatures from 37° to 100°C. The optimal pH of the enzyme was determined by running the standard assay for L-aspartate oxidase activity at 37°C using sodium acetate buffer (50 mM), potassium phosphate buffer (50 mM), Tris/HCl buffer (50 mM), and glycine/NaOH buffer (50 mM) for the pH ranges of 4–6, 6–8, 7.5–8.8, and 8.5–10.0, respectively. The thermostability was determined using the diluted enzyme (2.2 mg/ml pure enzyme was diluted 10 times with 50 mM potassium phosphate buffer, pH 7.5). The Michaelis constants were determined from Lineweaver-Burk plots (Cleland 1997) of data obtained from the initial rate of L-aspartate oxidation at 37°C.

Extraction and determination of flavin

The flavin compound from the enzyme was extracted with 1% (w/v) ice-cold perchloric acid. After removing the precipitate formed by centrifugation, the supernatant was used to identify the flavin compound by HPLC. An aliquot of the sample was subjected to a column (6.0 mm × 15 cm) of TSK gel ODS-80Ts (Tosoh) equilibrated with 10 mM potassium phosphate buffer, pH 6.0, containing 20% (v/v) methanol. The column was eluted with a 15-ml linear gradient of 20%–100% methanol in the same solution at a flow rate of 1.0 ml/min. The effluent from the column was monitored by a UV detector at the wavelength of 260 nm.

Results

Expression of the gene and purification of the recombinant enzyme

In the genome sequenced from *P. horikoshii*, we found a gene consisting of 1,395 base pairs of nucleotides, whose predicted amino acid sequence showed 35.1% identity with the *E. coli* L-aspartate oxidase [Fig. 1, National Center for Biotechnology Information (NCBI) Accession No. P10902] (Flachmann et al. 1988). *E. coli* JM109 transformed with p2496 exhibited hyperthermostable L-aspartate oxidase activity, which was not lost by incubation at 80°C for 60 min. Therefore, this protein was determined to be the L-aspartate oxidase from the *P. horikoshii*. The enzyme (hereafter referred to as the *P. horikoshii* L-aspartate oxidase) was purified to homogeneity from the extract of *E. coli* cells as described in the Materials and methods section. About 50 mg of the purified enzyme was obtained from 7 l of the *E. coli* culture. When the enzyme activity was measured with the standard assay for L-aspartate oxidase activity, a lack of linearity between the initial rate of the reaction and the amount of enzyme was observed (linearity was obtained only by using less than 0.6 μg of the enzyme in the reaction mixture). A similar phenomenon has also been observed for the *E. coli* L-aspartate oxidase (Nasu et al. 1982; Mortarino et al. 1996). It has been suggested that the decrease in activity observed at high enzyme concentrations is due to product inhibition by iminoaspartate because iminoaspartate forms a dead-end complex with L-aspartate oxidase (Mortarino et al. 1996). The specific activity of the purified *P. horikoshii* L-aspartate oxidase was estimated to be about 3.2 unit/mg at 37°C at an enzyme concentration below 0.6 μg in the reaction mixture.

SDS-PAGE of the purified enzyme gave only one band; the subunit molecular mass was determined to be about 51 kDa, and was consistent with the molecular weight (51,925) calculated from the amino acid sequence. The native molecular mass of the enzyme determined by HPLC was about 151 kDa (not shown). These results indicate that the *P. horikoshii* L-aspartate oxidase likely has a trimer structure. The N-terminal amino acid sequence of the purified enzyme was determined to be MMEMRVGIVGGGL

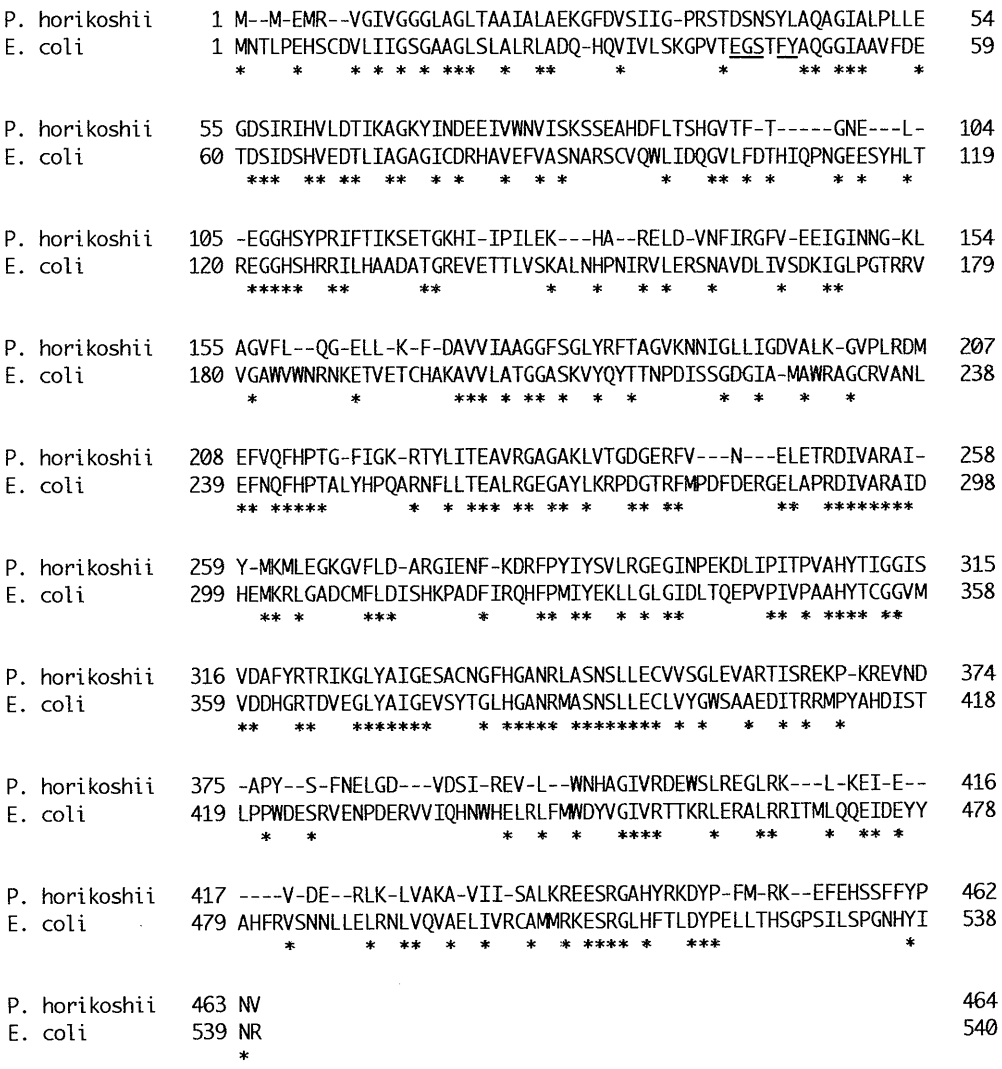

Fig. 1. Alignment of the amino acid sequence of L-aspartate oxidases from *Pyrococcus horikoshii* and *Escherichia coli*. *Asterisks* represent conserved residues among the two enzymes. The residues that are proposed to take part in the binding of the isoalloxazine portion of FAD in the *E. coli* enzyme are *underlined*

AGLTAAIALAEKGF, which was identical to the predicted sequence deduced from the open reading frame.

Characterization of the *P. horikoshii* L-aspartate oxidase

We were able to detect dye-linked L-aspartate dehydrogenase activity at temperatures from 37° to 100°C using ferricyanide as an electron acceptor (see below), and the optimum temperature was about 90°C (Fig. 2A). The optimum pH of the enzyme was 8.6 (data not shown). The enzyme also showed high thermostability; upon heating at 80°C for 60 min, the enzyme retained its full activity, but it lost 20% of its activity at 100°C after a 60-min incubation (Fig. 2B). Complete inactivation was observed upon incubation at 115°C for 10 min. The enzyme could be kept at a low temperature of around 4°C without any loss of activity for at least 2 months. As described for the *E. coli* L-aspartate oxidase (Nasu et al. 1982), the *P. horikoshii* enzyme also showed no classical Michaelis-Menten type kinetics for L-aspartate: linear double-reciprocal plots were obtained only at L-aspartate concentrations above 2 mM. The apparent *K*m value at L-aspartate concentrations between 2 and 20 mM was calculated to be 8.2 mM at 37°C.

Absorption spectra and prosthetic group

The purified enzyme did not lose its activity by dialysis against 10 mM potassium phosphate buffer (pH 7.0). The addition of 50 μM FAD or FMN to the dialyzed enzyme had no effect on the activity. The enzyme showed two pronounced absorption peaks at around 370 nm and 450 nm in addition to that at 280 nm (data not shown). This spectrum shows that the enzyme is a typical flavoprotein. The flavin compound in the purified enzyme was analyzed by HPLC as described in the Materials and methods section. The flavin compound was identified to be FAD and not FMN (data not shown). The cofactor of *E. coli* L-aspartate oxidase could be completely removed by dialysis against 1 M KBr for 1 day (Seifert et al. 1990). When the *P. horikoshii* enzyme was dialyzed against 3 M KBr and 0.5% charcoal for 3 days, about 58% of FAD still remained with the protein. Upon denaturation with 1% perchloric acid, almost all (98%) of the

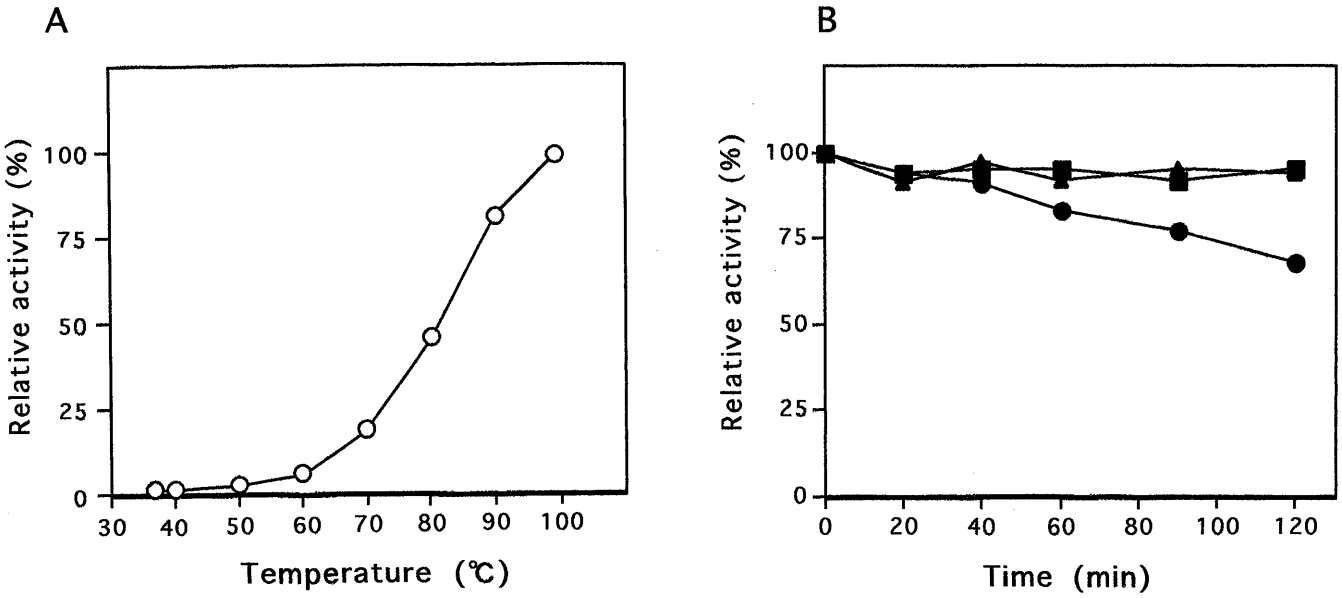

Fig. 2A,B. Characterization of *P. horikoshii* L-aspartate oxidase. **A** Temperature profile of dye-linked L-aspartate dehydrogenase activity of *P. horikoshii* L-aspartate oxidase. Ferricyanide was used as an electron acceptor. Activity at 100°C was defined as 100%. **B** Thermostability of *P. horikoshii* L-aspartate oxidase at 80°C (*triangles*), 90°C (*squares*), and 100°C (*circles*). Samples incubated at each temperature were taken at specific time intervals, and the residual activity was determined by the standard assay for L-aspartate oxidase activity at 37°C. Activity prior to heat treatment was defined as 100%

Table 1. Electron acceptor specificity of *P. horikoshii* L-aspartate oxidase

Electron acceptor	Relative activity (%)
Oxygen	100[a]
DCIP	237
PMS/INT	96
Ferricyanide	247
Fumarate	113

DCIP, 2,6-dichlorophenol-indophenol; PMS, phenazine methosulfate; INT, *p*-iodonitrotetrazolium violet
[a]Specific activity of oxygen was defined as 100%

absorbance of the enzyme at 450 nm was released into solution. These results indicate that FAD binds to the enzyme tightly but noncovalently.

Substrate and electron acceptor specificity

The ability of the *P. horikoshii* L-aspartate oxidase to catalyze the oxidation of various amino acids was examined using the standard assay for L-aspartate oxidase activity. The enzyme acted exclusively on L-aspartate. The following substrates were inert: L-alanine, L-arginine, L-asparagine, L-cysteine, L-glutamine, L-glutamate, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, D-aspartate, D-asparagine, and D-glutamate. The electron acceptor specificity of the enzyme was examined using the assay system for dye-linked L-aspartate dehydrogenase and L-aspartate: fumarate oxidoreductase. The reaction rate was determined for a range of enzyme concentrations in which the linearity between the initial rate of the reaction and the amount of enzyme was maintained. As shown in Table 1, ferricyanide (1.5 mM), DCIP (0.1 mM), and PMS (0.1 mM, final electron acceptor: 0.1 mM INT) exhibited electron acceptor activity. Ferricyanide was the most preferred electron acceptor of the enzyme. Similar to the *E. coli* enzyme, the *P. horikoshii* L-aspartate oxidase could utilize fumarate as an electron acceptor at a rate comparable to oxygen (Tedeschi et al. 1996) (Table 1).

Discussion

The predicted amino acid sequence of the *P. horikoshii* L-aspartate oxidase showed high sequence homology with the *E. coli* fumarate reductase (FRD-A, 28.5%) (GenBank Accession No. AAA23437) and succinate dehydrogenase (SDH-A, 28.9%) (GenBank Accession No. P10444) (data not shown). These are complex, membrane-bound multisubunit systems, the flavoproteins of which can catalyze fumarate reduction and succinate dehydrogenation coupled with electron transfer to or from other enzyme redox centers (Hägerhäll 1997). In an alignment of the sequences of the *E. coli* L-aspartate oxidase, FRD-A, and SDH-A, a considerable degree of identity (approximately 30%) and the presence of several conserved residues have been observed (Seifert et al. 1990). These observations indicate that the *P. horikoshii* and *E. coli* L-aspartate oxidases, FRD-A, and SDH-A might be evolutionarily related. In general, FRD-A and SDH-A are known to be able to utilize a variety of elec-

tron acceptors, including DCIP and PMS (Hägerhäll 1997). On the other hand, the *E. coli* L-aspartate oxidase has been described to show very low selectivity for the electron acceptor (Tedeschi et al. 1997). Besides oxygen and fumarate, only naphtho- or benzoquinones can be reduced at a comparable rate by the *E. coli* enzyme (Tedeschi et al. 1997). We showed that the *P. horikoshii* enzyme catalyzes L-aspartate dehydrogenation in the presence of an artificial electron acceptor such as PMS, DCIP, or ferricyanide. Therefore, it will be interesting to determine the structural difference in the electron acceptor binding site between the *E. coli* L-aspartate oxidase and the *P. horikoshii* enzyme.

Recently, Tedeschi et al. (1996) proposed that the *E. coli* L-aspartate oxidase can be considered as a particularly soluble fumarate reductase, and fumarate can act as the electron acceptor of the enzyme in the absence of oxygen. This feature is physiologically reasonable as it allows L-aspartate oxidase to be functional under both aerobic and anaerobic conditions. In facultative aerobic organisms such as *E. coli*, the de novo biosynthesis of NAD can take place both under aerobic and anaerobic conditions. We have now shown that L-aspartate oxidase is present in the obligate anaerobe *P. horikoshii*. The *P. horikoshii* enzyme utilized fumarate as the preferred electron acceptor in the absence of oxygen. Although the physiological significance of the oxidase activity of the enzyme is still not clear, it is possible to speculate that fumarate may act as the natural electron acceptor of the enzyme in *P. horikoshii*.

It has been reported that the binding of FAD to the *E. coli* L-aspartate oxidase is relatively weak (Seifert et al. 1990; Mortarino et al. 1996). The cofactor of the *E. coli* enzyme could be completely removed by exhaustive dialysis against 1 M KBr (Seifert et al. 1990). However, in the case of the *P. horikoshii* enzyme, about 58% of the cofactor still remained with the protein even after dialysis against 3 M KBr for 3 days. This shows that the binding of FAD to the *P. horikoshii* L-aspartate oxidase is extremely tight. Recently, the 3-D structure of the *E. coli* L-aspartate oxidase was determined (Mattevi et al. 1999). The X-ray analysis of the *E. coli* L-aspartate oxidase was performed using the FAD-free apoform of the enzyme, because crystals of the holoenzyme were not obtained even when an excess of FAD was added to the crystallization medium (Mattevi et al. 1999). This dissociation of FAD from the *E. coli* crystalline protein also indicates weak FAD-protein interactions. The FAD-binding site in the *E. coli* enzyme has been proposed from comparison with other flavoproteins that display the FAD-binding motif in a highly conserved manner (Mattevi et al. 1999) and from the results of mutagenesis studies (Mortarino et al. 1996). In the *E. coli* L-aspartate oxidase, Glu^{43}, Gly^{44}, Ser^{45}, Phe^{47}, and Tyr^{48} are proposed to take part in the binding of the isoalloxazine portion of FAD (Mortarino et al. 1996; Mattevi et al. 1999). Site-directed mutagenesis of these residues reduced the affinity for the coenzyme (Mortarino et al. 1996). In the *P. horikoshii* enzyme, these residues are replaced by Asp, Ser, Asn, Tyr, and Leu, respectively (Fig. 1). The structural difference associated with these substitutions may be related to the large difference in the dissociation properties of FAD in these enzymes.

In the de novo NAD biosynthetic pathway of prokaryotes (Fig. 3A), L-aspartate oxidase catalyzes the oxidation of L-aspartate to iminoaspartate, which is subsequently condensed with dihydroxyacetone phosphate by quinolinate synthase to form quinolinate. Quinolinate is then converted to the nicotinic acid mononucleotide by quinolinate phosphoribosyltransferase. Nicotinic acid mononucleotide reacts with ATP in a reaction catalyzed by nicotinamide mononucleotide adenylyltransferase to give nicotinic acid adenine dinucleotide, which is then amidated by NAD synthase to form NAD. On the basis of a similarity search of public databases, all the genes encoding the homologue of these enzymes were identified in the *P. horikoshii* genome. The deduced amino acid sequence from the nucleotide sequence of the open reading frames PH0013, PH0011, PH0464, and PH0182, which is assigned in the *P. horikoshii* OT-3 genome (Kawarabayasi et al. 1998), showed 35%, 34%, 59%, and 32% identity with the *E. coli* quinolinate synthase (NCBI Accession No. P11458) (Flachmann et al.

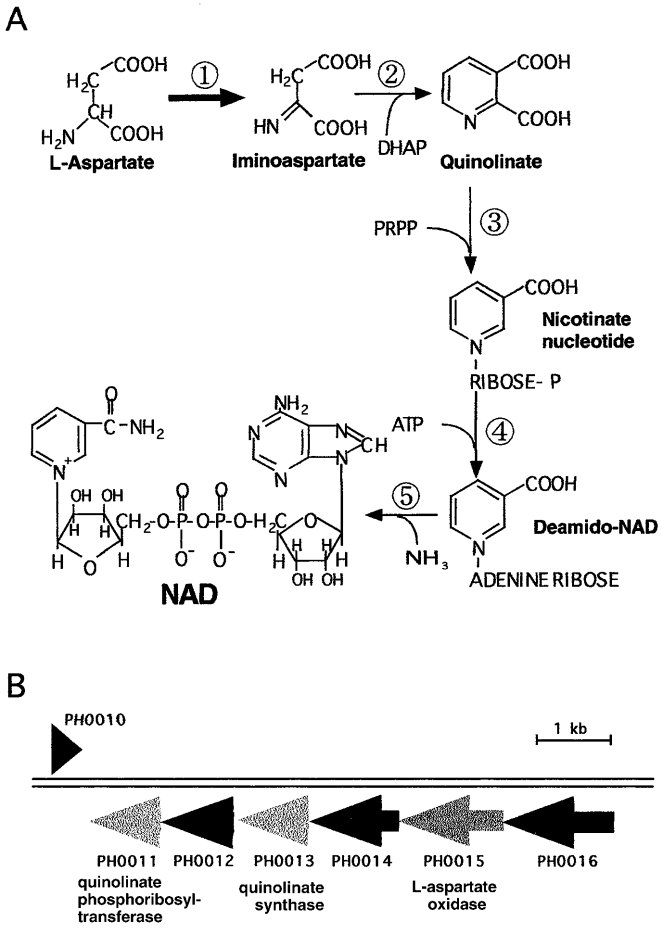

Fig. 3. **A** De novo nicotinamide adenine dinucleotide (NAD) biosynthetic pathway of prokaryotes. The *circled numbers* correspond to the following enzymes: *1*, L-aspartate oxidase; *2*, quinolinate synthase; *3*, quinolinate phosphoribosyltransferase; *4*, nicotinamide mononucleotide adenylyltransferase; *5*, NAD synthase. *DHAP*, dihydroxyacetone phosphate; *PRPP*, 5-phospho-D-ribosyl-1-pyrophosphate. **B** Genetic organization of the L-aspartate oxidase gene (PH0015) region in the *P. horikoshii* genome. The locations and orientations of the genes are indicated by *arrows*

1988), the *E. coli* quinolinate phosphoribosyltransferase (NCBI Accession No. AAC36922) (Bhatia and Calvo 1996), the *Methanococcus jannaschii* nicotinamide mononucleotide adenylyltransferase (NCBI Accession No. Q57961) (Raffaelli et al. 1997), and the *E. coli* NAD synthase (NCBI Accession No. BAA15529) (Allibert et al. 1987), respectively. The L-aspartate oxidase gene (PH0015) formed a cluster with a putative quinolinate synthase gene (PH0011) and a putative quinolinate phosphoribosyltransferase gene (PH0013) together with three other unknown genes (PH0012, PH0014, and PH0016) (Fig. 3B). Although the functional analysis of those genes has not yet been done, our observations strongly suggest that *P. horikoshii* may use the de novo NAD biosynthetic pathway under anaerobic conditions, and the L-aspartate oxidase may function in catalyzing the first reaction of NAD biosynthesis in this organism. To elucidate the entire aspect of the de novo NAD biosynthetic pathway in *P. horikoshii*, the expression of the other four genes, PH0013, PH0011, PH0464, and PH0182, in *E. coli* and a functional analysis of their products are currently in progress.

Acknowledgments This study was supported in part by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science and the New Energy and Industrial Technology Development Organization (NEDO) project promoted by the Ministry of International Trade and Industry of Japan.

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