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Discovery and Characterization of a Thermostable NADH Oxidase from *Pyrococcus horikoshii* OT3

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A gene (PH0311) encoding a hypothetical protein from the genome sequence data of the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3 was cloned and over-expressed in *Escherichia coli*. The purified recombinant protein was found to possess FAD-dependent NADH oxidase activity, although it lacked sequence homology to any other known general NADH oxidase family. The product of the PH0311 gene was thus designated *Ph*NOX (NADH oxidase from *Pyrococcus horikoshii*), with an estimated molecular weight of 84 kDa by gel filtration and 22 kDa by SDS-PAGE, indicating it to be a homotetramer of 22 kDa subunits. *Ph*NOX catalyzed the oxidation of reduced β-NADH with subsequent formation of H_2O_2 in the presence of FAD as a cofactor, but not α-NADH, α-NADPH, or β-NADPH. *Ph*NOX showed high affinity for β-NADH with a K_m value of 3.70 μM and exhibited optimum activity at pH 8.0 and 95 °C as it is highly stable against high temperature.

Key Words: H_2O_2-forming NADH oxidase, *Pyrococcus horikoshii*, Homotetramer, Thermostability, PH0311

Introduction

NADH oxidase (NADH: oxygen oxidoreductase, NOX; EC 1.6.99.3) catalyzes the oxidation of NADH to NAD^+ through reduction of oxygen. The NOXes are divided into two types dependent upon the mode of reduction. One catalyzes a two-electron reduction of O_2 to H_2O_2; the other a four-electron reduction of O_2 to H_2O.[1] Although oxygen is toxic for obligate anaerobes, several enzymes catalyzing NADH-dependent reduction of oxygen have been found in anaerobes.[2,3] The physiological role of NOX is diverse, depending upon its substrates and products in different organisms. The NOX derived from aerobically grown *Streptococcus faecalis* catalyzes the direct four-electron reduction of O_2 to two molecules of H_2O_2.[4-7] However, the NOX isolated from *Bacillius megaterium* produces H_2O_2 upon consumption of oxygen in a two-electron reduction.[8] Moreover, other NOXes produced either H_2O or superoxide.[1,9] In these organisms, the oxidation of NADH was considered to be not only the defense mechanism to reduce traces of oxygen and reestablish anaerobiosis, but also the regeneration system of NAD^+.[10] Some thermophile-derived NOXes have been purified from *Pyrococcus furious*, *Thermus* aquaticus, and *Thermotoga maritima* and proposed to protect anaerobes from oxidative stress.[11-13] However, the functions of NOXes from thermophiles are unknown and remain to be elucidated.[14]

Pyrococcus horikoshii is a hyperthermophilic archaeon with an optimum growth temperature at 98 °C. Most hyperthermophilic archaea are strict anaerobes by fermentative-type metabolism and grow optimally near 100 °C, depending on the reduction of elemental sulfur for growth.[15] The genome sequencing of *P. horikoshii* was completed at the National Institute of Technology and Evaluation in Japan[16] and from the sequence data, the PH0311 gene was suggested to be a candidate enzyme for the aromatic ring hydroxylation activity as judged by homolog searches in the NCBI database. Until now, there have been no reports concerning the product of this gene. Therefore, we have cloned and expressed this gene in *Escherichia coli*.

Here we report the identification of the PH0311 gene from *P. horikoshii* OT3 as NOX and the biochemical characterization of its expressed protein.

Materials and Methods

Materials. The pET-26b(+) expression vector and *E. coli* strain BL21 Star (DE3) used in this study were supplied by Novagen (Madison, WI, USA). Restriction enzymes and DNA-modifying enzymes were obtained from Takara Shuzo (Otsu, Shiga, Japan). α-NADH, α-NADPH, β-NADH, β-NADPH, FAD, IPTG, kanamycin, cytochrome *c* from bovine heart, superoxide dismutase and catalase were purchased from Sigma-Aldrich (St. Louis, MO, USA). The synthesis of DNA primers was performed by COSMO Genetech (Seoul, Korea). All chemicals and reagents used were commercially available and of the highest reagent grade.

Cloning of the PH0311 gene and construction of expression plasmid. The complete sequence of *P. horikoshii* OT3 has been reported.[17] Standard cloning techniques were used throughout. The PH0311 gene for the aromatic ring hydroxylation enzyme was discovered using a BLAST search and amplified by the polymerase chain reaction (PCR). The sequences of the PCR primers were: 5'-GGAATTCCATATGGTTATGAAAGAAG-ACATTGCAACG-3' (upper primer, containing an *Nde* I digestion sequence as underlined); 5'-CCGCTCGAGAAGCATT-CCAAGCTTTCTTTTAGCTTC-3' (lower primer, containing

Abbreviations: NADH, nicotinamide adenine dinucleotide reduced form; NOX, NADH oxidase; *Ph*NOX, NOX from *Pyrococcus horikoshii*; FAD, flavin adenine dinucleotide.

an *Xho* I digestion sequence as underlined). The PCR product was cloned into an expression vector pET-26b(+) using *Nde* I and *Xho* I sites. The resulting vector was designated as pET-6× His-PH0311 and was transformd into the *E. coli* strain BL21 Star (DE3). The colony containing the appropriate insert was identified by DNA sequencing.

Expression and purification of the recombinant enzyme. The *E. coli* strain BL21 Star (DE3) harboring each of the constructed plasmids was grown in LB broth containing kanamycin (50.0 μg/mL) at 37 °C and an overexpression was induced at OD_{600} = 0.9 - 1.0 with 1.0 mM IPTG for 4 h. The induced cells were harvested by centrifugation at 10,000 g for 10 min at 4 °C and resuspended in 20.0 mM Tris-HCl buffer (pH 7.9) containing 5.0 mM EDTA and 100.0 μM phenylmethanesulfonyl fluoride. The resuspended cells were lysed by sonication for approximately 10 min using an ultrasonic processor (Sonics & Materials Inc., Newtown, CT, USA). The disrupted cell suspension was heated at 80 °C for 30 min to denature the *E. coli* proteins. After the aggregated proteins were removed by centrifugation at 12,000 g for 30 min, the supernatant was collected and stored at 4 °C.

The supernatant containing the enzyme bearing the C-terminal His-tag was subject to affinity column chromatography with Ni-conjugated agarose (His-bind Buffer kit, Novagen, Gibbstown, NJ, USA). The column was washed with 20.0 mM Tris-HCl buffer (pH 7.9) containing 0.5 M NaCl and 60.0 mM imidazole; the enzyme was then eluted with 20.0 mM Tris-HCl buffer (pH 7.9) containing 0.5 M NaCl and 1.0 M imidazole.[18] The pool of active fractions was concentrated by ultrafiltration and dialyzed against 50.0 mM potassium phosphate buffer (pH 7.0). The purified enzyme was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli.[19]

Native molecular size determination. The native molecular mass was measured by size exclusion chromatography on an FPLC system (Pharmacia Biotech, Piscataway, NJ, USA) at room temperature. The enzyme was applied to a Superdex® 200 HR 10/30 fast protein liquid chromatography column (Pharmacia Biotech, Piscataway, NJ, USA) equilibrated with 50.0 mM potassium phosphate buffer (pH 7.0). The native molecular mass of the enzyme was determined by comparison to the following molecular size standards: β-amylase (200 kDa); yeast alcohol dehydrogenase (150 kDa); bovine serum albumin (66 kDa); trypsinogen (24 kDa); cytochrome *c* (12.4 kDa) (Sigma-Aldrich).

Enzyme assay. NOX activity was measured spectrophotometrically under aerobic conditions at room temperature by monitoring the oxidation of β-NADH at 340 nm (ε_{340} = 6.22 mM^{-1} cm^{-1}) with a HITACHI U-2000 double-beam spectrophotometer (Hitachi CO., Tokyo, Japan) using cuvettes of 1.0 cm path-length (Sigma-Aldrich). The reaction mixture contained the following, in a final volume of 1.0 mL: 50.0 mM potassium phosphate buffer (pH 7.0); 150.0 μM β-NADH; 150.0 μM FAD; an appropriate amount of enzyme. The reaction was then initiated by addition of the prepared enzyme. One unit of activity was defined as the amount of enzyme catalyzing the oxidation of 1.0 μmol of β-NADH to β-NAD^+ per min. To measure the kinetic parameters, reaction rates were measured at a series of NADH concentrations by fitting the data to Lineweaver-Burk plots. The parameters (with standard deviation) were determined by five separate experiments. Protein concentration was determined by the method of Bradford using γ-globulin as the standard protein.[20]

Substrate specificity of the enzyme toward various substrates. Oxidation reactions of the enzyme toward α-NADH, α-NADPH, and β-NADPH, through consumption of oxygen, were measured under standard reaction conditions described for β-NADH to test as alternative electron donors.

Determination of hydrogen peroxide and superoxide formation in the enzyme reaction. The production of hydrogen peroxide in the enzyme reaction was determined by a spectrophotometrical method.[14] In this method, formation of a dye compound by electron transfer from H_2O_2 was detected using catalase. Superoxide production in the enzyme reaction was determined by monitoring cytochrome *c* reduction by superoxide. The reaction mixture contained the following, in a final volume of 1.0 mL: 0.2 mM β-NADH; 20.0 mM cytochrome *c*; 0.3 mM EDTA; 10 μg of the enzyme incubated with FAD. The reaction was initiated by addition of 50 units of superoxide dismutase and monitored at 550 nm.

Absorption spectra of the enzyme. The absorption spectra of the enzyme and the enzyme preincubated with FAD were measured by a HITACHI U-2000 double-beam spectrophotometer at room temperature for 10 min. The unbound FAD was removed by dialysis from the enzyme solution preincubated with FAD. A solution containing 1.5 mg of the enzyme was introduced into cuvettes of 1.0 cm path-length and stoppered for absorption spectra measurements (200 - 600 nm).[21]

Optimal temperature and pH of the enzyme. The optimal temperature of the enzyme was determined in the 50.0 mM potassium phosphate buffer (pH 7.0) at temperatures ranging from 25 to 100 °C, at a concentration of 0.1 mg/mL. Effects of pH on enzymatic activity were determined using the following buffers (50.0 mM) at the indicated pH: citrate-sodium phosphate buffer, from pH 4.0 to 6.0; potassium phosphate buffer, from pH 6.0 to 8.5; Tris-HCl buffer, from pH 8.5 to 9.5; glycine-NaOH buffer, from pH 9.5 to 10.5.

Thermostability of the enzyme. The thermostability of the enzyme was measured at 90 and 95 °C. The enzyme was incubated in 50.0 mM potassium phosphate buffer (pH 7.0) from 1 - 6 h. After chilling the sample on ice, residual activity was determined under standard conditions.

Results and Discussion

Expression of the *P. horikoshii* PH0311 gene and identification of the protein. In the genome sequence data of *P. horikoshii*, it was discovered that the open reading frame PH0311, composed of 531 base pairs, encodes a protein homologous with the aromatic ring hydroxylation enzyme from *Thermoplasma volcanium* GSS1 (55% identity), as judged by homolog searches in the NCBI database (Fig. 1). This PH0311 gene was cloned by PCR and the amplified gene ligated into the expression vector pET-26b(+) for generating the recombinant plasmid pET-6× His-PH0311. *E. coli* strain BL21 Star (DE3) cells were transformed by the recombinant plasmids and transformant cells cultivated at 37 °C in Luria-Bertani medium. Induction of the

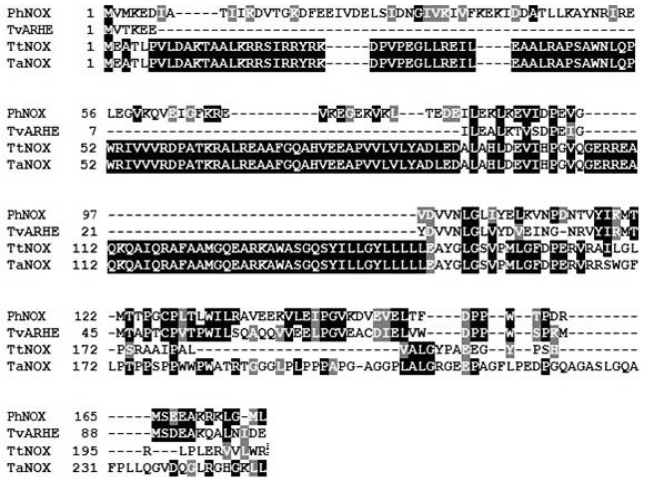

Figure 1. Aligned amino acid sequences of *Ph*NOX, *Tv*ARHE, *Tt*NOX, and *Ta*NOX. The abbreviations of the sources of the enzymes are: *Ph*-NOX, NADH oxidase from *Pyrococcus horikoshii* (GenBank accession number, NP_142296); *Tv*ARHE, aromatic ring hydroxylation enzyme from *Thermoplasma volcatium* (NP_111912); *Tt*NOX, NADH oxidase from *Thermus thermophilus* (YP_143691); *Ta*NOX, NADH oxidase from *Thermus aquaticus* (S23449). The sequences have been aligned with dashes that indicate gaps. The consensus identical (black) or similar (gray) amino acids are shaded.

Table 1. Purification of the recombinant *Ph*NOX

Step	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)	Purification (fold)
Crude Extract	223	2342	10.5	100	1
Heat Treatment	23	1219	53	52	5
IMAC	12	1008	84	43	8

Enzyme activity was determined using β-NADH and a unit of activity is defined as the amount of enzyme catalyzing the formation of 1.0 μmol of product, β-NAD^+, per min under the conditions of the specific assay.

recombinant protein was performed by cultivating *E. coli* until an optical density at 600 nm reached approximately 1.0, following the addition of IPTG. The histidine-tagged recombinant protein was efficiently purified in 8-fold at a yield of 43% by heat treatment and Ni-agarose affinity chromatography (Table 1). The purified protein gave a single, electrophoretic band in the presence of SDS, Fig. 2A.

The aromatic ring hydroxylase activity of this recombinant protein, consisting of 176 amino acids, was examined using various aromatic substrates.[22] No activity was detected using these substrates. Thorough experimentation on the enzymatic activity of this protein revealed its ability to catalyze the oxidation of β-NADH through reduction of oxygen. However, the protein had no sequence homology to any other known general NOX family, including NOXes from hyperthermophilic archaea (below 14% identity, Fig. 1). When the protein was preincubated with FAD, the oxidation of β-NADH proceeded rapidly (Table 2). The oxidation reaction of the protein toward β-NADH was barely detectable in the absence of FAD. To confirm the protein binding to FAD, a light absorbance analysis

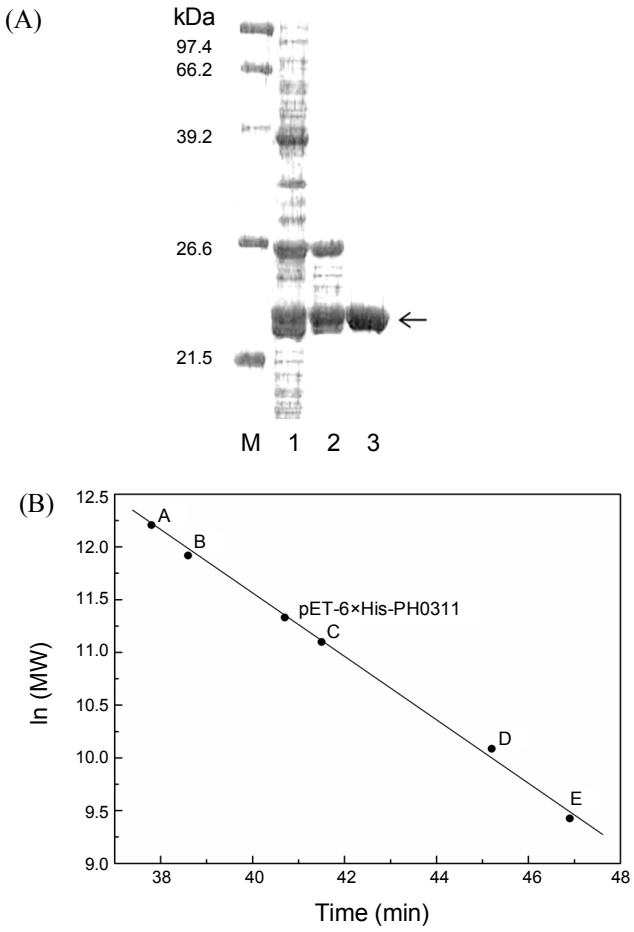

Figure 2. Molecular mass determination of the recombinant pET-6×His-PH0311. (A) SDS-PAGE analysis at different stages of purification. Lane M, molecular weight standard marker; lane 1, cell extract after sonication; lane 2, supernatant after heat treatment (heating at 80 °C for 30 min); lane 3, purified pET-6×His-PH0311 (arrow) after Ni-agarose affinity chromatography. (B) Molecular mass determination of the native recombinant pET-6×His-PH0311 by gel filtration analysis. X axis is elution time of proteins and Y axis is plotted by calculating to ln(molecular mass). A, β-amylase (200 kDa); B, yeast alcohol dehydrogenase (150 kDa); C, bovine serum albumin (66 kDa); D, trypsinogen (24 kDa); E, cytochrome *c* (12.4 kDa).

was performed. The purified protein showed typical flavin absorption spectra with peaks at 375 and 455 nm after preincubation with FAD (Fig. 3). Conversely, the nascent protein expressed in *E. coli* did not show the flavin spectra, therefore the recombinant protein produced from the pET-6×His-PH0311 is a novel FAD-dependent NOX that catalyzes the oxidation reaction of NADH to NAD^+ and is thus designated *Ph*NOX (NADH oxidase from *P. horikoshii*).

Quaternary structure determination of the *Ph*NOX. Enzyme's quaternary structure was analyzed by SDS-PAGE and gel filtration. Comparison of the relative mobility of the *Ph*NOX with a standard protein indicated an approximate molecular mass of 22 kDa by SDS-PAGE analysis (Fig. 2A). However, gel filtration analysis under nondenaturing conditions showed the *Ph*NOX to possess a molecular mass of approximately 84 kDa (Fig. 2B), indicating *Ph*NOX to be a homotetramer of 22 kDa subunits and the smallest NOX among NOXes thus reported

Table 2. Substrate specificity of the *Ph*NOX

Substrate	Cofactor	Specific Activity (U/mg)
α-NADH	-	ND
α-NADH	0.5 mM FAD	ND
β-NADH	-	ND
β-NADH	0.5 mM FAD	84.35 ± 0.03
α-NADPH	-	ND
α-NADPH	0.5 mM FAD	ND
β-NADPH	-	ND
β-NADPH	0.5 mM FAD	ND

Enzyme activity was determined under standard reaction condition except FAD incubation time for 5 h. ND, not detected. Values are means ± S.D., generally based on n ≥ 5.

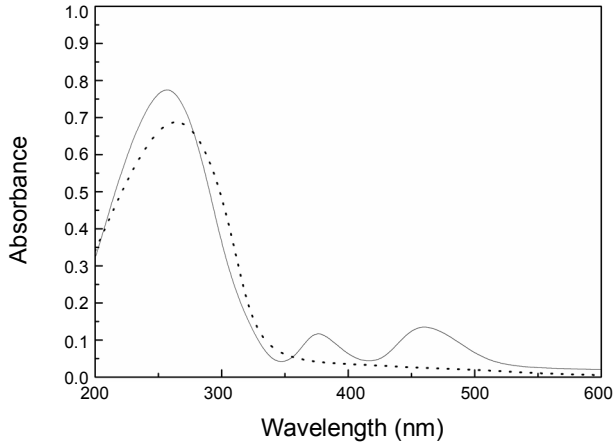

Figure 3. Visible spectra of the holo-*Ph*NOX and apo-*Ph*NOX. Solid line: The holo-*Ph*NOX containing FAD (solid line) showed a typical flavin spectrum with peaks at 375 and 455 nm. However, the apo-*Ph*NOX (dot line) did not show the flavin spectrum with peaks at 375 and 455 nm. Absorbance was measured in 50.0 mM potassium phosphate buffer (pH 7.0) at 25 °C.

from an anaerobic organism. This quaternary structure of the *Ph*NOX also differs from most NOXes from anaerobic organisms possessing two or more identical subunits with molecular masses between 100 and 450 kDa. A NOX from *Thermus thermophilus* was composed of two identical subunits as determined by X-ray crystallography,[23] while a NOX from *Eubacterium* was determined to be a homotrimer of 72 kDa subunits by gel filtration chromatography.[24] The NOXes from *Clostridium thermohydrosulfurium* and *Thermococcus profundus* were a homohexamer of 71 kDa subunits and 49 kDa subunits, respectively.[9,25]

Catalytic properties of the PhNOX. The catalytic properties of the *Ph*NOX were investigated with the NADH oxidation reaction. The optimum pH of the *Ph*NOX was 8.0 at 80 °C (Fig. 4A). The *Ph*NOX showed less than 60% of its maximum activity below pH 6.0 and above 9.5. A similar result has been found in the NOXA-1 of hyperthermophilic archaeon *Archaeoglobus fulgidus* (pH 8.0).[14] Nevertheless, a pH optimum near neutrality has been observed for NOXes from *Thermotoga hypogea* (pH 7.0), *Clostridium aminovalericum* (pH 7.0), and *Eubacterium*

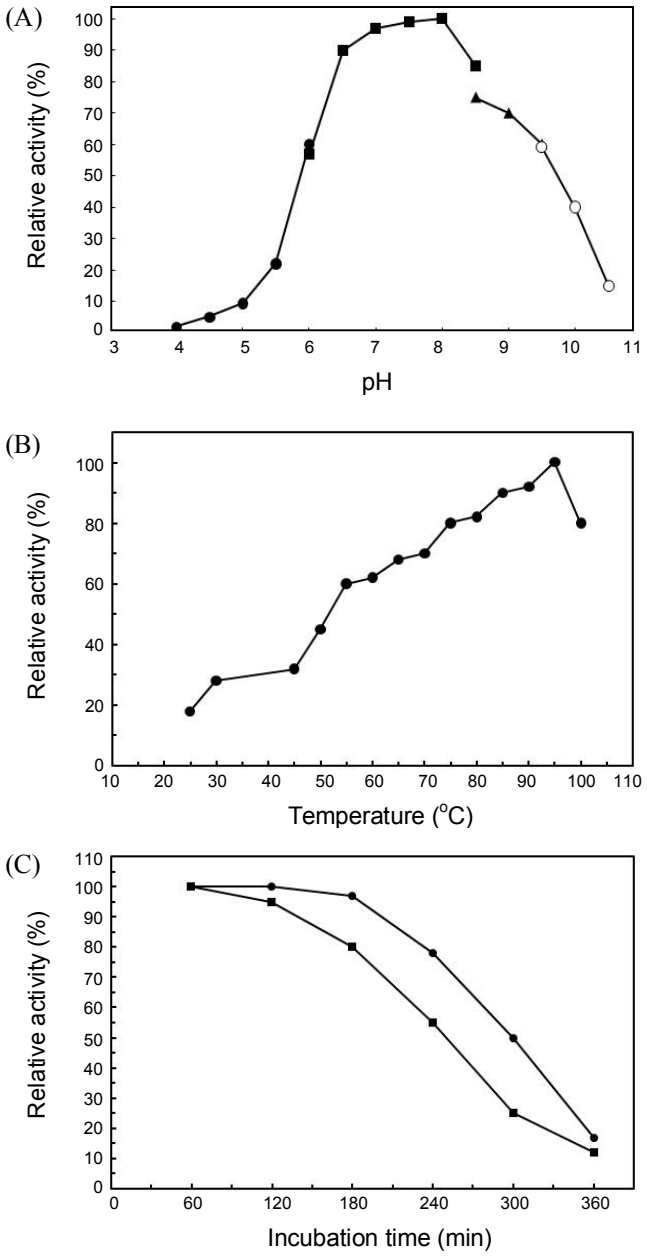

Figure 4. Effect of pH, temperature, and heating on enzyme activity of the *Ph*NOX. (**A**) Effects of pH on enzyme activity were determined using the following buffers at the indicated pH. pH 4.0 - 6.0: 50.0 mM citrate-sodium phosphate buffer (-●-); pH 6.0 - 8.5: 50 mM potassium phosphate buffer (-■-); pH 8.5 - 9.5: 50.0 mM Tris-HCl buffer (-▲-); pH 9.5 - 10.5: 50.0 mM glycine-NaOH buffer (-○-). (**B**) Effects of temperature on enzyme activity were determined in 50.0 mM potassium phosphate buffer (pH 7.0) at temperatures ranging from 25 to 100 °C. (**C**) Effects of heating on enzyme activity were measured at 90 and 95 °C. Symbols: -●- and -■- for the remaining activity of the sample incubated at 90 and 95 °C, respectively.

ramulus (pH 7.0).[9,26,27,28] In order to understand the effects of temperature on enzyme activity, the reaction mixture was incubated from 25 to 100 °C. The optimum temperature of the *Ph*NOX was 95 °C (Fig. 4B). The *Ph*NOX showed approximately 80% of its maximum activity, even at 100 °C, but showed less than 50% of its maximum activity below 50 °C. The *Ph*NOX activity showed a preference for high temperatures expected

of an enzyme from a hyperthermophile.

The *Ph*NOX was specific for β-NADH as the reducing substrate with a specific activity of 84.0 U/mg (Table 2). Compared to the NOXes from *Clostridium thermohydrosulfuricum* (28.0 U/mg), *Eubacterium sp.* VPI12708 (48 U/mg), and *Thermus thermophilus* HB8 (52.0 U/mg), the *Ph*NOX was more active.[9,24,29] However, oxidation reactions of the *Ph*NOX toward α-NADH, α-NADPH, and β-NADPH were undetectable. This substrate specificity of the *Ph*NOX was similar to that of a NOX from *Lactococcus lactis*.[30] To further determine the function of the *Ph*NOX, kinetic parameters of the enzyme with β-NADH at pH 8.0 were measured. The K_m and k_{cat} values of the *Ph*NOX were 3.70 ± 0.03 μM and 115 ± 3 s^{-1}, respectively. The *Ph*NOX also showed an affinity for β-NADH higher than other NOXes from: *Archaeoglobus fulgidus* NOXB-1, with a K_m of 11.0 μM; *Clostridium thermohydrosulfurium*, with a K_m of 19.0 μM; *Eubacterium ramulus*, with a K_m of 38.0 μM; *Thermotoga hypogea*, with a K_m of 7.5 μM.[9,14,26,28]

NOXes can be divided into two major categories. One group produces H_2O_2 from O_2 while the other group yields H_2O. To clarify to which type the *Ph*NOX belongs, the reduction of molecular oxygen was examined. β-NADH oxidation catalyzed by *Ph*NOX resulted in the stoichiometric production of H_2O_2 confirmed by the spectrophotometrical methods.[14] This result demonstrated that the *Ph*NOX belongs to the H_2O_2 producing type. However, β-NADH oxidation catalyzed by *Ph*NOX did not result in the formation of the superoxide radical, confirmed by a cytochrome *c* reduction assay. This indicates that the electron transfer during β-NADH oxidation by the *Ph*NOX occurs mainly from β-NADH, directly to the electron acceptor O_2, not *via* a superoxide radical intermediate.

Thermal stability of the PhNOX. The thermostability of the *Ph*NOX toward the β-NADH oxidation reaction was investigated by incubation of the enzyme at 90 °C and another at 95 °C. The half-lives for thermal inactivation of the *Ph*NOX were approximately 5 h at 90 °C and 4 h at 95 °C, respectively (Fig. 4B). The results described above indicate that the *Ph*NOX has high thermal stability and is expected to be applicable as a biocatalyst for NAD^+ recycling.

In conclusion, we expressed the hypothetical protein of the PH0311 gene from *P. horikoshii* in *E. coli* and characterized the purified recombinant protein. The hypothetical protein first reported herein is a novel flavin-dependent NADH oxidase (*Ph*NOX) that catalyzes the oxidation of β-NADH with the formation of hydrogen peroxide as an end product. The *Ph*NOX appears to be distinct from NOXes of other sources reported, particularly regarding amino acid sequence, molecular weight, quaternary structure, reaction specificity, kinetic parameters, and stability, although common features can be pointed out as mentioned above. Further studies are in progress to elucidate the detailed physiological functions of this enzyme.

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