Full Paper

Metal chaperone, NhpC, involved in the metallocenter biosynthesis of nitrile hydratase

(Received March 7, 2020; Accepted March 24, 2020; J-STAGE Advance publication date: November 6, 2020)

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Pseudomonas chlororaphis B23 yields nitrile hydratase (NHase) used for the production of 5-cyanovaleramide at the industrial level. Although the *nhpC* gene (known as *P47K*) located just downstream of the NHase structural genes (*nhpAB*) has been important for efficient NHase expression, the key role of *nhpC* remains poorly studied. Here, we purified two NHases expressed in the presence and absence of *nhpC*, respectively, and characterized them. The purified NHase expressed with *nhpC* proved to be an iron-containing holo-NHase, while the purified one expressed without *nhpC* was identified as an apo-NHase, which was iron-deficient. These findings indicated that *nhpC* would play a crucial role in the post-translational incorporation of iron into the NHase active site as a metal chaperone. In the overall amino acid sequence of NhpC, only the N-terminus exhibited similarities to the CobW protein involved in cobalamin biosynthesis, the UreG and HypB proteins essential for the metallocenter biosynthesis of urease and hydrogenase, respectively. NhpC contains a P-loop motif known as a nucleotide-binding site, and Lys23 and Thr24 are conserved in the P-loop motif in NhpC. Expression analysis of NHase formed in the presence of each mutant NhpC (i.e., K23A and T24A) resulted in immunodetectable production of a mutant NhpC and remarkable expression of NHase lacking the enzyme activity. These findings suggested that an intact P-loop containing Lys23 and Thr24 would be essential for the NhpC function *in*

vivo for the post-translational metallocenter assembly of NHase.

Key Words: apoenzyme; holoenzyme; iron

Introduction

Nitriles (R–C≡N) are organic compounds with a triple bond between their carbon and nitrogen atoms. Most nitrile compounds are highly toxic to almost all living organisms and are quite difficult to degrade. In nature, however, some bacteria have the ability to convert nitriles directly into non-toxic or less toxic compounds. Previous studies revealed the existence of two different pathways for the microbial degradation of nitrile compounds (Asano et al., 1982; Kobayashi et al., 1992a; Yamada et al., 1979). First, nitrilase catalyzes the hydrolysis of nitriles into carboxylic acids and ammonia (Kobayashi et al., 1993; Komeda et al., 1996). Second, nitrile hydratase (NHase) catalyzes the hydration of nitriles to amides (Kobayashi et al., 1995), which are then hydrolyzed to carboxylic acids and ammonia by amidase (Kobayashi et al., 1997). Furthermore, the resulting acids are metabolized into acyl-CoAs by acyl-CoA synthetase (Abe et al., 2008; Hashimoto et al., 2005). Finally, nitriles are assimilated as a carbon and nitrogen source for bacterial growth (Sakashita et al., 2008).

NHases are metalloenzymes, and classified into Fe-types and Co-types according to the metal involved (Kobayashi and Shimizu, 1998). Both Fe-type and Co-type NHases composed of α -subunits and β -subunits are post-

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None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.

Fig. 1. Organization of the NHase gene clusters of *P. chlororaphis* B23 and *Rhodococcus* sp. N-774.

translationally modified. Metal ions in both Fe-NHases and Co-NHases are located in their α -subunits, which share a characteristic metal-binding motif [CXLC(SO₂H)SC(SOH)] containing two oxidized cysteine residues: cysteine-sulfinic acid (α Cys-SO₂H) and cysteine-sulfenic acid (α Cys-SOH). In the holo- α -subunit, the post-translationally oxidized Cys-SO₂H and Cys-SOH have been deprotonated to Cys-SO₂⁻ and Cys-SO⁻, forming salt bridges with the two arginines of the β -subunit (which are conserved in all known Fe-type and Co-type NHases) in the holo-NHase (Hourai et al., 2003; Huang et al., 1997; Miyanaga et al., 2001; Nagashima et al., 1998). The apoenzyme is metal-deficient and the corresponding cysteine residues are likely to be non-oxidized, as shown by the results of previous studies on NHase (Miyanaga et al., 2004).

Previously, we discovered a novel post-translational activation mechanism for the Co-type NHase of *Rhodococcus rhodochrous* J1, which is an important industrial microorganism, producing acrylamide and nicotinamide (Kobayashi et al., 1992b; Lan et al., 2017). The incorporation of Co into Co-type NHase depends on α -subunit exchange between Co-free apo-NHase and a NHase maturation mediator (complex of Co-containing α -subunit and chaperone protein) (Sun et al., 2016; Zhou et al., 2008, 2009). We named this unexpected mechanism “self-subunit swapping”.

On the contrary, there is little information on the mechanism of post-translational maturation of Fe-type NHase. *Pseudomonas chlororaphis* B23, one of the Fe-type NHase-producers, has a gene cluster involved in the nitrile metabolism comprising eight genes: *nhpR*, *oxdA* (known as *P38K*), *amiA*, *nhpA*, *nhpB*, *nhpC* (known as *P47K*), *nhpS* (known as *orfE*), and *acsA* (Fig. 1) (Nishiyama et al., 1991; Sakashita et al., 2008). *nhpA* and *nhpB* encode the α -subunit and β -subunit of the NHase, respectively. These genes are significantly induced by the addition of methacrylamide to the medium and the amount of NHase produced makes up more than 50% of total soluble protein in *P. chlororaphis* B23. We have discovered the overall “nitrile pathway” (aldoxime → nitrile → amide → acid → acyl-CoA) comprising four enzymes, i.e., aldoxime dehydratase (OxdA), Fe-type NHase, amidase (AmiA), and acyl-CoA synthetase (AcsA) (Hashimoto et al., 2005). Although the structures of Fe-type and Co-type NHases are very similar, their post-translational activation mechanisms are thought to be quite different from each other. While the gene coding for the Co-type NHase chaperone protein is not found in the gene cluster, *nhpC* required for efficient NHase expression is located just downstream of the *nhpAB* genes. The *E. coli* transformant

harboring plasmid pPCN4, which carries the NHase genes (*nhpAB*) and three other ORFs (*oxdA*, *amiA*, and *nhpC*), yields an active recombinant NHase (Nishiyama et al., 1991). Because *oxdA* and *amiA* code for aldoxime dehydratase and amidase, respectively (Oinuma et al., 2003), they are not involved in the formation of NHase in *E. coli*. Whereas *nhpC* has been found to be important for the functional expression of NHase, as NHase activity is not detected in the *E. coli* transformant harboring plasmid pPCN7, which carries *oxdA*, *amiA*, and *nhpAB* but not *nhpC* (Nishiyama et al., 1991). However, the amount of NHase produced is very low, being detectable only by the immunological method. Thus, the role of *nhpC* in the formation of active NHase remains poorly understood. Here, our studies have revealed that NhpC participates in the incorporation of Fe into the NHase as a metal chaperone.

Materials and Methods

Expression of the recombinant Nhases. The *nhpABC* genes were amplified by PCR with pPCN4 as a template. The following two oligonucleotide primers were used: a sense primer (*nhpA-S*), 5'-GAATTCTAAGGAGGAATATGCATATGAGTACATCTATTTCCACG-3', containing an NdeI recognition site (underlined); and an antisense primer (*nhpC-AS*), 5'-GGATCCTCAAGTCGGTTGAACCGCCATG-3', containing a BamHI recognition site (underlined). The amplified DNA was subcloned into vector pHSG398 and checked by DNA sequencing. The insert DNA was digested with NdeI and BamHI, and then inserted into the respective sites of pET-24a(+). The resultant plasmid was designated as pET-*nhpABC*; in this construct, the *nhpABC* genes were under the control of the T7 promoter. As for the construction of pET-*nhpAB* that harbors the *nhpAB* gene only, an antisense primer (*nhpB-AS*), 5'-GGATCCTCATGCGTTCACCGCTTGGG-3', containing a BamHI recognition site (underlined), was used instead of the *nhpC-AS* antisense primer for PCR.

Escherichia coli BL21(DE3) was transformed with pET-*nhpABC* or pET-*nhpAB*, and the recombinant cells were used for the overproduction and purification of the recombinant NHase. The transformed cells were incubated with reciprocal shaking at 37°C in 10 ml of 2 × YT medium containing 50 µg/ml kanamycin. After overnight cultivation, the entire culture was inoculated into 0.5 liters of 2 × YT medium containing 50 mg/l FeSO₄·7H₂O and 50 µg/ml kanamycin, followed by incubation with shaking at 37°C for 1.5 h. IPTG was then added to a final concentration of 0.1 mM to induce the T7 promoter, and further cultivation was carried out at 15°C for 24 h.

Purification of the recombinant NHase(nhpABC). All purification steps were carried out at 0–4°C. HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-KOH (pH 7.2) containing 22 mM *n*-butyric acid and 20% glycerol was used throughout the purification. Centrifugation was performed for 20 min at 16,200 × *g*.

Step 1: Preparation of cell-free extracts. Washed cells from 0.5 l of culture broth were suspended in 100 mM HEPES-KOH (pH 7.2) containing 22 mM *n*-butyric acid and 20% glycerol, and then disrupted by sonication at 200 W for 20 min with an Insonator model 201 M (Kubota, Tokyo).

The cell debris was removed by centrifugation.

Step 2: Ammonium sulfate fractionation. The resultant supernatant was fractionated with ammonium sulfate (30–60% saturation), followed by dialysis against 10 mM HEPES-KOH (pH 7.2) containing 22 mM *n*-butyric acid and 20% glycerol.

Step 3: DEAE-Sephacel column chromatography. The dialyzed solution was applied to a DEAE-Sephacel column (200 ml; GE Healthcare, Chicago, IL, USA) equilibrated with 10 mM HEPES-KOH (pH 7.2) containing 22 mM *n*-butyric acid and 20% glycerol. Protein was eluted by increasing KCl linearly from 0 to 0.6 M in the same buffer. The active fractions were collected, and then ammonium sulfate was added to give 65% saturation. The precipitate was collected by centrifugation, dissolved in 100 mM HEPES-KOH (pH 7.2) containing 22 mM *n*-butyric acid and 20% glycerol, and then dialyzed against 10 mM HEPES-KOH (pH 7.2) containing 22 mM *n*-butyric acid and 20% glycerol.

Step 4: Resource Q column chromatography. The dialyzed solution was applied to a Resource Q column (6 ml; GE Healthcare) equilibrated with 10 mM HEPES-KOH (pH 7.2) containing 22 mM *n*-butyric acid and 20% glycerol. Protein was eluted by increasing KCl linearly from 0 to 0.5 M in the same buffer. The homogeneity of the purified protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of the recombinant NHase(*nhpAB*). The following steps were altered for purification of NHase(*nhpAB*). After SDS-PAGE, the fractions containing the 25 kDa band were collected. As for ammonium sulfate fractionation, cell-free extracts were fractionated with ammonium sulfate (10–30% saturation).

Enzyme assays. All of the reactions were performed under linear conditions with an appropriate amount of protein and a suitable reaction time, unless otherwise noted. The standard assay mixture comprised 30 mM potassium phosphate buffer (KPB) (pH 7.2), 100 mM propionitrile and an appropriate amount of enzyme in a total volume of 500 μ l. The reaction was started by the addition of the enzyme and carried out at 10°C for 5 min. The reaction was stopped by the addition of 250 μ l methanol to the reaction mixture, and a supernatant was obtained by centrifugation (23,300 $\times$ g, 10 min). The amount of propionamide formed in the reaction mixture was determined by gas chromatography with a Shimadzu GC-9A system (Kyoto, Japan) equipped with a flame ionization detector, a 2.1-m $\times$ 3.2-mm (ID) glass column packed with Propak type PS (80/100 mesh; Waters, Inc., Milford, MA, USA) being used. The temperatures of the injector and column were 250 and 190°C, respectively. One unit of the NHase activity was defined as the amount of the enzyme that catalyzed the production of 1 μ mol propionamide/min under the above-described conditions. The protein concentrations were determined with a Nacalai Tesque protein assay kit (Nacalai Tesque Inc., Kyoto) using bovine serum albumin as the standard by the method of Bradford (1976).

Molecular mass determination. The purified enzyme sample was applied to a Superose 6 HR10/30 column (GE

Healthcare), which was attached to an ÄKTA purifier (GE Healthcare), and then eluted with 10 mM HEPES-KOH (pH 7.2) containing 22 mM *n*-butyric acid, 20% glycerol and 0.15 M KCl. The absorbance of the effluent was recorded at 280 nm. The molecular mass of the enzyme was calculated from the mobilities of the standard proteins, i.e., glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa), and cytochrome *c* (12.4 kDa).

Metal analysis. All glassware was soaked in 1 M HCl overnight and then exhaustively rinsed with distilled water before use. Prior to analysis, the enzyme was dialyzed against 1 mM KPB (pH 7.0). An enzyme sample containing 2 mg/ml was analyzed with an inductively coupled argon plasma spectrophotometer, ICAP-575 (Nippon Jarrel-Ash Co. Ltd., Kyoto).

Circular dichroism (CD) analysis. CD measurements were carried out with a Jasco spectropolarimeter, model J-720W (Japan Spectroscopic Company, Tokyo), equipped with a thermal incubation system at 20°C, with a 0.1-cm light path cell. CD measurements of the purified enzyme and each mutant were carried out with a 0.1-cm light path cell at the protein concentration of 0.2 mg/ml in 10 mM KPB (pH 7.0) in the far-UV region (200–260 nm). The ellipticity in the CD spectra was normalized as to the protein concentration.

Electrophoresis. SDS-PAGE was performed in a 12% polyacrylamide slab gel according to Laemmli (1970). The gel was stained with Coomassie brilliant blue R-250. The relative molecular mass of the enzyme subunit was determined from the relative mobilities of marker proteins, i.e., phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α -lactalbumin (14.4 kDa).

Construction of pET-*nhpABC-H*. The *nhpC* gene lacking the stop codon was amplified by PCR with pET-*nhpABC* as a template. The following two oligonucleotide primers were used: a sense primer (*nhpC*-S), 5'-CATATGATTGAAGGCGCCAGGC-3', containing an NdeI recognition site (underlined); and an antisense primer (*nhpC*-His-AS), 5'-GTCGACAGTCGGTTGAACCGC-CATGG-3', containing a SalI recognition site (underlined). The amplified DNA was subcloned into vector pHSG398 and checked by DNA sequencing. The insert DNA was digested with AatII (which cleaves the unique restriction site located within the *nhpC* gene) and SalI, and then inserted into the AatII and XhoI sites of pET-*nhpABC*. The resultant plasmid was designated as pET-*nhpABC-H*; in this construct, *nhpC* was expressed as a C-terminally His-tagged NhpC.

Site-directed mutagenesis. Site-directed mutagenesis was carried out on *nhpC* by means of the overlap extension PCR protocol (Ho et al., 1989; Pogulis et al., 1996). To construct the K23A mutant, two PCRs, with plasmid pET-*nhpABC* as the template, were performed with primer pairs *nhpC*(K23A)-S (5'-CCTCGGCGCCGGCGCA-ACCACCCTGCTC-3') plus T7T and A1 (5'-TCTGCAGCCTGTGCTCCTGC-3') plus *nhpC*(K23A)-

AS (5'-GCGTTGAGCAGGGTGGTTGC-GCCGGCGCCG-3'). These reactions produced 3' and 5' fragments of *nhpC*, respectively, whose sequences overlapped by 25 base pairs at the mutation. A second round of PCR was performed by mixing equimolar amounts of the first round products, followed by amplification between primers A1 and T7T. The second-round product was digested with *ScaI* and *NcoI*, inserted into the corresponding sites of pET-*nhpABC*-H, and then sequenced. A clone with the sequence of the desired K23A mutation was chosen and transformed into *E. coli* BL21(DE3). The T24A mutation was carried out in the same manner as the K23A mutation, with internal primer pairs *nhpC*(T24A)-S (5'-CTCGGCGCCGGCAAAGCCACCCTGCTCAAC-3') plus *nhpC*(T24A)-AS (5'-GCGTTGAGCAGGGTGGC-TTTGCCGGCGCC-3').

Fig. 2. SDS-PAGE of NHase(*nhpABC*) and NHase(*nhpAB*) samples at various stages of purification.

Protein bands were detected by staining with Coomassie Brilliant Blue. The arrow indicates the band corresponding to NHase. Lanes 1–4, NHase(*nhpABC*); lanes 5–8, NHase(*nhpAB*). Lane M, marker proteins; lane 1, cell-free extract; lane 2, ammonium sulfate (30–60% saturation); lane 3, DEAE-Sephacel chromatography; lane 4, Resource Q chromatography; lane 5, cell-free extract; lane 6, ammonium sulfate (10–30% saturation); lane 7, DEAE-Sephacel chromatography; lane 8, Resource Q chromatography.

Western blot analysis. Western blotting analysis was carried out as described below. Proteins were separated by SDS-PAGE under reducing conditions and then electroblotted onto PVDF membranes (Sequi-Blot™, Bio-Rad Laboratories, Inc., Hercules, CA, USA). After blocking with 5% (w/v) skim milk, the blots were incubated with Penta-His™ antibody (Qiagen, Inc., Hilden, Germany) at room temperature for 1 h and goat anti-mouse IgG-alkaline phosphatase conjugate (IgG-AP) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 h. The immunoreactive proteins were visualized with the colorimetric substrate BCIP/NBT (Nacalai Tesque Inc.).

Fig. 3. Determination of the molecular masses of NHase(*nhpABC*) and NHase(*nhpAB*) by gel filtration.

Marker proteins used for gel filtration: glutamate dehydrogenase (yeast) (290 kDa); lactate dehydrogenase (pig heart) (142 kDa); enolase (yeast) (67 kDa); myokinase (yeast) (32 kDa); and cytochrome *c* (horse heart) (12.4 kDa). The molecular masses determined are shown by open circles.

Table 1. Purification of NHase expressed in the presence of NhpC.

Step	Total protein mg	Total activity units	Specific activity units/mg	Yield of protein %	Yield %
Cell-free extract	1890	432000	233	100	100
Ammonium sulfate (30–60% saturation)	928	409000	440	49.2	94.6
DEAE-Sephacel	540	311000	575	28.9	71.9
Resource Q	113	133000	1180	6.0	30.7

Table 2. Purification of NHase expressed in the absence of NhpC.

Step	Total protein mg	Total activity units	Specific activity units/mg	Yield of protein %
Cell-free extract	1190	N.D.	N.D.	100
Ammonium sulfate (10–30% saturation)	379	N.D.	N.D.	31.8
DEAE-Sephacel	31	N.D.	N.D.	2.6
Resource Q	8.0	N.D.	N.D.	0.672

N.D.: Not detected.

Fig. 4. Far-UV CD spectra of NHase(*nhpABC*) and NHase(*nhpAB*). Solid line, the purified NHase(*nhpABC*); and dashed line, the purified NHase(*nhpAB*).

Results

Role of *nhpC* for functional NHase expression

We here constructed two plasmids, pET-*nhpABC* and pET-*nhpAB*. The former contains NHase structural genes *nhpAB* and *nhpC*, but lacks *oxdA* and *amiA* genes, while the latter contains only NHase structural genes. *E. coli* cells harboring pET-*nhpAB* (grown in the presence of iron) were used to express NHase under the same conditions as for *E. coli* cells harboring pET-*nhpABC*. The bands of the α - and β -subunits of NHase were obvious on SDS-PAGE (Fig. 2, lane 5), while NHase activity in cell-free extracts of the *E. coli* transformant was not detected. On the contrary, the amount of NHase expressed using *E. coli* cells harboring pET-*nhpABC* was similar to that in the case of *nhpAB* (Fig. 2, lane 1), and remarkable activity (233 units/mg) was observed in the cell-free extracts.

To determine the differences between the NHases expressed from pET-*nhpABC* and pET-*nhpAB*, each of the corresponding two enzymes was completely purified and characterized. Hereafter, we refer to the NHases expressed from pET-*nhpABC* and pET-*nhpAB* as NHase(*nhpABC*) and NHase(*nhpAB*), respectively. On SDS-PAGE, NHase(*nhpABC*) and NHase(*nhpAB*) each gave only one 25 kDa band corresponding to the molecular masses of 22,118 and 24,546 Da calculated from deduced amino acids of NhpA and NhpB, respectively (Fig. 2, lanes 4 and 8). The two purified enzymes had identical N-terminal amino acid sequences of (S/M)-(T/D)-(S/G)-(I/F)-(S/H)-(T/D)-(T/L). These are the same as the deduced amino acid sequences [(M)STSISTT] derived from *nhpA* and those (MDGFHDL) from *nhpB*. NHase(*nhpABC*) was found to be an $\alpha_2\beta_2$ heterotetramer (approximately 82 kDa), whereas NHase(*nhpAB*) was found to be an $\alpha\beta$ heterodimer (approximately 47.6 kDa) (Fig. 3). Compared with the specific activity of 1180 units/mg shown by NHase(*nhpABC*) (Table 1), NHase(*nhpAB*) exhibited no activity at all (Table 2). The iron content, which is essential for the activity (Nagashima et al., 1998), of NHase(*nhpABC*) was found to be 0.91 mol/mol of $\alpha\beta$, whereas that of NHase(*nhpAB*) was low (0.21 mol/mol of $\alpha\beta$). NHase(*nhpABC*) and NHase(*nhpAB*) showed different far UV CD spectra to each other (Fig. 4), indicating

A

NHase(*nhpABC*) NHase(*nhpAB*)

B

C

Fig. 5. Spectroscopic analyses of NHase(*nhpABC*) and NHase(*nhpAB*).

A. Photograph of the purified enzyme solutions of NHase(*nhpABC*) and NHase(*nhpAB*). B. UV-vis spectra of NHase(*nhpABC*) and NHase(*nhpAB*). Solid line, the purified NHase(*nhpABC*); and dashed line, the purified NHase(*nhpAB*). C. The difference spectrum generated by subtraction of the spectrum of NHase(*nhpAB*) from that of NHase(*nhpABC*).

that the secondary structure of the two enzymes were different from each other. The purified NHase(*nhpABC*) was pale green in solution (Fig. 5A), while the purified NHase(*nhpAB*) was colorless. The UV-Vis spectrum of NHase(*nhpABC*) was very similar in the shape and position to that of NHase purified from *P. chlororaphis* B23 (Nagasawa et al., 1987). The absorption spectrum of NHase(*nhpAB*) in the visible range differed completely

Fig. 6. Analyses of the production of NHase and C-terminally His-tagged NhpCs.

A. SDS-PAGE analysis of recombinant NHase expressed with a C-terminally His-tagged NhpC and its mutants. The arrow indicates the band corresponding to NHase. Lane M, marker proteins; lane 1, cell-free extract of pET-*nhpABC*-H/*E. coli* BL21(DE3); lane 2, cell-free extract of pET-*nhpABC*(K23A)-H/*E. coli* BL21(DE3); and lane 3, cell-free extract of pET-*nhpABC*(T24A)-H/*E. coli* BL21(DE3). B. Western-blot analysis of a C-terminally His-tagged NhpC and its mutants. The arrow indicates the band corresponding to the C-terminally His-tagged NhpC. Lane M, marker proteins; lane 1, cell-free extract of pET-*nhpABC*-H/*E. coli* BL21(DE3); lane 2, cell-free extract of pET-*nhpABC*(K23A)-H/*E. coli* BL21(DE3); and lane 3, cell-free extract of pET-*nhpABC*(T24A)-H/*E. coli* BL21(DE3).

from that of NHase(*nhpABC*), showing no peak at 720 nm (Figs. 5B and 5C). The change in absorbance at 720 nm showed good correlation with the disappearance of the enzyme activity, and a similar difference absorption spectrum with a positive peak at 710 nm correlated with the decrease in enzyme activity through binding of endogenous nitric oxide (NO) in the iron center of NHase was observed (Odaka et al., 1997). These findings demonstrated that the NHase expressed from pET-*nhpAB* was an apo-NHase that is iron-deficient, in contrast to the iron-containing holo-NHase expressed from pET-*nhpABC*, and that *nhpC* was essential for the post-translational maturation of NHase *in vivo*.

Construction of a C-terminally His-tagged NhpC

On SDS-PAGE, the NhpC protein band was not found around the molecular mass of 46,668 Da calculated from deduced amino acids of NhpC (Fig. 2, lane 1). In order to detect the formation of NhpC, we constructed pET-*nhpABC*-H to express a C-terminally His-tagged NhpC. By SDS-PAGE, we analyzed cell-free extracts prepared from the transformant, but found no obvious NhpC band at the 47 kDa position (Fig. 6A, lane 1). On Western blot analysis using Penta-HisTM antibody, a band derived from C-terminally His-tagged NhpC was immunologically detected (Fig. 6B, lane 1). The amount of NHase on SDS-PAGE and the NHase activity (258 units/mg) in the cell-free extracts from pET-*nhpABC*-H/*E. coli* BL21(DE3) were similar to those in the case of pET-*nhpABC*/*E. coli* BL21(DE3). These findings indicated that the formation of NhpC expressed under the T7 promoter was very low, but enough for the production of functional NHase, and that *in vivo* NHase activation by the NhpC was not affected by the addition of His-tag to NhpC.

Mutational analysis of C-terminally His-tagged NhpC

The N-terminal region of the deduced amino acid se-

quences of NhpC exhibited sequence similarity with some proteins belonging to the ABC ATPase superfamily. NhpC contained a P-loop motif (Walker A motif), a Walker B motif that is the site of magnesium binding (Sydor et al., 2013), a highly conserved cysteine-rich (CXCC) motif known as a metal-binding site in other metal chaperones (Cheng et al., 2013; Lu et al., 2003), and a guanine-binding motif (NKED guanine-binding motif) (Fig. 7). In the P-loop motif in NhpC, Lys23 and Thr24 known to be essential for a nucleotide-binding site in a variety of other proteins (Mehta et al., 2003; Moncrief and Hausinger, 1997; Saraste et al., 1990) were conserved. Thus, we constructed two mutant NhpCs (i.e., K23A and T24A) by site-directed mutagenesis. According to the same procedure as used for *E. coli* cells harboring pET-*nhpABC*, pET-*nhpABC*(K23A)-H was expressed in *E. coli* in the presence of iron, and then characterized. By SDS-PAGE and Western analyses, the remarkable production of the α - and β -subunits of NHase and the production of NhpC(K23A) were confirmed, respectively (Fig. 6). NHase activity in cell-free extracts of the *E. coli* transformant was completely absent. As for pET-*nhpABC*(T24A)-H, the production of NHase and NhpC(T24A), and the NHase activity were identical to those of pET-*nhpABC*(K23A)-H. These findings indicated that Lys23 and Thr24 were crucial amino acid residues for the post-translational activation of NHase by NhpC.

Discussion

NHase of *P. chlororaphis* B23, which was previously used as a catalyst for acrylamide manufacture (Ryuno et al., 1988; Yamada and Kobayashi, 1996), has been used for the production of 5-cyanovaleramide, a herbicide intermediate, at the industrial level (Hann et al., 1999). As for the expression of the NHase gene in an *E. coli* host, its structural gene and adjacent regions are required for effi-

	Walker A motif (P loop motif)	
NhpC	MIEGAQAGRLPVTLSG-FLGAGKTTLLNAILRNR-QGLRVAVIVNDMSEVNLDAESVQRDVSLHGRDE	68
Orf1188	MVD---TRLPTVLSG-FLGAGKTTLLNEILRNR-EGRRVAVIVNDMSEINIDSAEVEREISLSRSEEE	64
CobW	MTTARANQKIPATVITG-FLGAGKTTMIRNLLQNA-DGKRIGLIINEFGDLGVDG-DVLKGCAGEACTED	68
UreG	MNSYKHPLRVGPGVSGKTALLEALCKAMRDTWQLAVVTNDIYTKEDQR-ILTEAGALAPERI	64
HypB	MCTTCGCGENLYIEGDEHNPHSAFRSAPFAPAARPK-MKITGIKAPEFTPSQTEEGDLHYGHGEAGTHAP	70
	Metal-binding site	Walker B motif
NhpC	-LIEMSGCICCTLRADLLEQISDLA-RQQR- ---DYLLIESTGISEPMPVAETFAFLDTGFSLSLARLD	135
Orf1188	KLVEMTNGCICSTLR-DLLSEISALA-RSGRF- ---DYLLIESTGISEPLPVGETFTFIDTDGHALSSPD-ST	130
CobW	DIIELTNGCICCTVADDFIPTMTKLLERENR- ---DHIIETSGLALPQLIAAFNWPDIR-SEVTVGCVT	136
UreG	VGVETG-GCPHTAIREADSMNLA AVEALSEKFGNLDLIFVESGGDNL SATFSP ELADLTIIYVIDVAEGE- ---	132
HypB	GMSQRRMLEVIDVLDKNNRLAERNRARFAARKQLVLNLVSSPGSGKTTLLTETLMRLKDSVPCAVIEG- ---	139
	Guanine-binding motif	
NhpC	TLVTVDGSGFQALLLESTDTVARADTEHTSTRHLADLLIEQVEYANVILVNKRDLIDEPGYQAVHAILAG-	206
Orf1188	PWSPLVDAN- ---SFLRDYTAGGRVEADAPEDERDIADLLVDQIEFADVILVSKADLISAPAPGRIDGVLRS-	198
CobW	VVDSAAVAAGRFAADHDKVDALRVEDDNL DHE SPI EEL FEDQLTAADLIVLNKTDLIDASGLKAVRDEVSSR	208
UreG	-----KIPRKGPGITKSDFLVINKTDLAPYVG- ---ASLEVMAS-	168
HypB	-----DQQTVNDAARIRATGTPAIQVNTGKGCHLDAQMIADAAPRLP	181
NhpC	LNPSARIMPMAGHNVALLSSLDTHLFDLPSLAASPGWMRKMEATDTPASESDTYGVTSWVYRERAPFHPQRL	278
Orf1188	LNEPLRLFDDGLGRIPLDITDGLFSLEKAAQAPGWLQELQGEHTPETEEVRWCTASTSHPSTPNVCMTSS	270
CobW	TSRKPTMIEAKNGEVAAILLLGLGVGTESDIANRK- ---SHHEMEHEAGEEHDHDEFDSFVVELGSIADPAAF	277
UreG	---DTQRMGRDPWTFTNLKQGDGLSTIIAFLDKGMLGK	205
HypB	LDDNGILFIENVGNLVCPSFADLGEKHKVAVLSVTEGEDKPLKYPHMAAASLMLLNKVDLLPYLNFDVEKC	253
NhpC	LEFLQKPWHNGRLRLSKGYFWLASRHLEIGLLAQSGKQFQWDYVGRWWNFIEPSQWPRDEYRLQGIMAKWDS	350
Orf1188	AASGPTESYSG-PRRSTGMTRAGSPRSDY- ---FQAGHLIRHGYVEPLVEVLPRDEWPADDYRRDGILDKWEE	338
CobW	IDRLKGVI AEHDVLR LKGFADVP- ---KPMRLLIQAVGARIDQYYDRAWGAGEKR- ---	329
UreG		205
HypB	IACAREVNPEIEIILIS- ---ATSGEGMDQWLNWLETQRCA	290
NhpC	VVGDCRQELVFIGQLDTRVLQRELDHCLLSAQEIAAGPLAWQALPAATAFDTEALSARPTPPMAVQPT	419
Orf1188	PVGDCRQELVFIGAIDPSLHRELDACLLTAEIELGPR- ---RVDHLERPPGAGYTDQTV	396
CobW	-----GTRLVVI- ---LHDMDEAAVRAAITLV- ---	354
UreG		205
HypB		290

Fig. 7. Alignment of the amino acid sequences of NhpC and metal chaperones.

Designations: NhpC from *P. chlororaphis* B23, Orf1188 from *Rhodococcus* sp. N-774, CobW from *Pseudomonas denitrificans*, UreG from *Klebsiella aerogenes*, and HypB from *Escherichia coli*.

cient expression of NHase activity (Nishiyama et al., 1991). The upstream region containing *oxdA* and the amidase structural gene (*amiA*) is required for maximum expression of NHase genes. On the other hand, lack of the downstream region containing *nhpC* causes a small level of NHase production, which is detectable only by the immunological method, and no NHase activity. Later, we found that *oxdA* encodes aldoxime dehydratase catalyzing the dehydration of aldoximes to nitriles (Konishi et al., 2006; Nomura et al., 2013; Oinuma et al., 2003). As for *Rhodococcus* sp. N-774, an Fe-type NHase-producer, the NHase gene and the downstream region are also necessary for production of the active NHase in both *Rhodococcus rhodochrous* and *E. coli* hosts (Hashimoto et al., 1994). In the case of an *E. coli* host, the recombinant NHase expressed without the downstream region is accumulated as inclusion bodies, and is not formed in the soluble fraction. The downstream region contains a single open reading frame (Orf1188) showing some level of amino acid sequence similarity (44%) with NhpC (Figs. 1 and 7). In the previous experiments on the *P. chlororaphis* B23 and *Rhodococcus* sp. N-774 NHases, each NHase expressed lacking the downstream region could not be purified because of less or no amount of the produced NHase. Based on the results of the enzyme assay for NHase activity and the expression of NHase in cell-free extracts from transformants expressed in the presence or absence of the

downstream region, the importance of *nhpC* or Orf1188 located in the region necessary for active NHase production was concluded (Fig. 1). In order to exclude the possibility of contributing factor(s) other than *nhpC*, here, an expression plasmid harboring the *nhpAB* genes (pET-*nhpAB*) and an expression plasmid harboring the *nhpABC* genes (pET-*nhpABC*) were constructed and used.

E. coli carrying pET-*nhpABC* showed the production of a large amount of active NHase (Fig. 2, lane 1). Unlike in previous studies (Nishiyama et al., 1991), *E. coli* carrying pET-*nhpAB* produced a large amount of inactive NHase in the soluble fraction (Fig. 2, lane 5). These findings made it possible to purify the NHase expressed in the absence of the *nhpC* gene. However, the NHase(*nhpAB*) was unstable, and almost of the NHase(*nhpAB*) proteins was precipitated during dialysis. We attempted to improve the instability of the NHase(*nhpAB*), but good conditions for stabilization were not determined. Thus, the yield of the NHase(*nhpAB*) during purification was quite low (0.672%) (Table 2). The purified NHase(*nhpAB*) was stable at 4°C for a few days.

In this study, we succeeded in purifying two enzymes expressed in the presence and absence of the *nhpC* gene, respectively, and performed a comparative study of them. There were the following several differences between NHase(*nhpAB*) and NHase(*nhpABC*). NHase(*nhpAB*) consisted of an $\alpha\beta$ heterodimer, as opposed to the $\alpha_2\beta_2$

heterotetramer of NHase(*nhpABC*) (Fig. 3). The color of the enzyme solution, and UV-Vis and CD spectra of NHase(*nhpABC*) were different from those of NHase(*nhpAB*), indicating that the two enzymes were spectroscopically different from each other (Figs. 4 and 5). Iron-deficient NHase(*nhpAB*) exhibited no NHase activity, whereas NHase(*nhpABC*) showing high NHase activity contained iron (Tables 1 and 2). Taking these results and observations together, NHase(*nhpABC*) and NHase(*nhpAB*) are a holo-enzyme and an apo-enzyme, respectively. These findings indicated that *nhpC* is responsible for *in vivo* incorporation of iron into the NHase active site as a metal chaperone.

To purify and characterize NhpC, we constructed a C-terminally His-tagged NhpC and found that the addition of the His-tag to the C-terminal of NhpC had no influence on its own expression or post-translational activation of apo-NHase. First, the cell-free extract prepared from pET-*nhpABC*-H/*E. coli* BL21(DE3) was applied to a nickel-chelating column of HisTrapTM HP (5 ml) (GE Healthcare) and eluted with a linear gradient (0–0.5 M) of imidazole. However, SDS-PAGE revealed that the C-terminally His-tagged NhpC was not present in any eluted fractions. These results indicated that the C-terminally His-tagged NhpC was not specifically bound to the affinity column or was not stable. Next, we attempted to purify NhpC by means of ammonium sulfate fractionation and several column chromatographies such as on DEAE Sephacel (200 ml; GE Healthcare), ResourceQ (6 ml), HiLoad 16/60 Superdex 200 prep grade (120 ml; GE Healthcare), and HiTrap Heparin HP (1 ml; GE Healthcare). Fractions containing the C-terminally His-tagged NhpC were detected by Western analysis. Unfortunately, NhpC could not be purified because the protein was eluted broadly and unstable.

Interestingly, only the N-terminus of NhpC exhibited similarities in amino acid sequence to many ATP- and GTP-binding proteins: in particular, the CobW protein from *Pseudomonas denitrificans*, the UreG protein from *Klebsiella aerogenes*, and the HypB protein from *E. coli* (Fig. 7). UreG and HypB are GTPases, being essential for the nickel incorporation into urease and hydrogenase, respectively (Maier et al., 1993; Moncrief and Hausinger 1997). CobW is probably involved in cobalamin biosynthesis as a metal (cobalt) chaperone (Crouzet et al., 1991). There was no similarity in the overall sequence except in the above limited region. The higher similar regions among them were the NTPase domain defined by Walker A (P-loop) and the Walker B motif. The P-loop consists of a Gly-rich sequence followed by a conserved Lys and a Ser or Thr, and the corresponding Lys23 and Thr24 were conserved in NhpC. To assess the importance of the NhpC P-loop motif, mutagenesis experiments were carried out to replace either of two conserved residues with Ala. On immunoblot analysis, each mutant NhpC was detectable in the soluble fraction (Fig. 6B). The *E. coli* transformant formed a large amount of the α - and β -subunits of NHase (Fig. 6A), but did not exhibit NHase activity at all. These findings indicated that the intact P-loop motif in NhpC was likely to be essential for the post-translational iron incorporation into the metallocenter of NHase. Recently,

the Fe-type NHase activator protein from *Rhodococcus equi* TG328-2 was purified and characterized. To provide ample amounts of a soluble form of the activator protein, this structural gene is synthesized with optimized *E. coli* codon usage, and overexpressed in *E. coli* as a maltose-binding protein (MBP) fused protein for rapid purification. Furthermore, optimal conditions for stabilization of the resultant activator protein, from which the MBP tag was removed, were determined. Finally, the Fe-type NHase activator protein proved to be a GTPase (Gumataotao et al., 2017). Since the deduced amino acid sequence of NhpC exhibited similarity with that of the activator protein, NhpC appeared to play a role in NHase metallocenter assembly through GTP hydrolysis. To date, however, *in vitro* post-translational maturation of the apoenzyme of Fe-NHase by the activator protein has not been successful. Further research on this Fe-type NHase metal chaperone is needed and will provide us with novel knowledge on NHase metallocenter assembly.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT). YH was in part supported by the Japan Science and Technology Agency, ERATO (JPMJER1502). The authors thank Dr. Toshihide Sakashita (University of Tsukuba) for the help in the determination of the N-terminal amino acid sequences of the purified enzymes. Special thanks are also due to Professor Kentaro Shiraki (University of Tsukuba) for the use of the CD spectrometer.

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