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Functional Expression of Nitrile Hydratase in *Escherichia coli*: Requirement of a Nitrile Hydratase Activator and Post-Translational Modification of a Ligand Cysteine[1]

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The nitrile hydratase (NHase) from *Rhodococcus* sp. N-771 is a photoreactive enzyme that is inactivated on nitrosylation of the non-heme iron center and activated on photo-dissociation of nitric oxide (NO). The nitrile hydratase operon consists of six genes encoding NHase regulator 2, NHase regulator 1, amidase, NHase α subunit, NHase β subunit and NHase activator. We overproduced the NHase in *Escherichia coli* using a T7 expression system. The NHase was functionally expressed in *E. coli* only when the NHase activator encoded downstream of the β subunit gene was co-expressed and the transformant was grown at 30°C or less. A ligand cysteine, αCys112, of the recombinant NHase was also post-translationally modified to a cysteine-sulfinic acid similar to for the native NHase. Although another modification of αCys114 could not be identified because of the instability under acidic conditions, the recombinant NHase could be reversibly inactivated by nitric oxide.

Key words: hydration, metalloprotein, nitrile, overexpression, post-translational modification.

Nitrile hydratase (NHase; EC 4.2.1.84) catalyzes the hydration of nitriles to the corresponding amides ($RCN + H_2O \rightarrow RCONH_2$) (*1*). The enzyme is well-known as one of the most industrially successful enzymes. It is currently used for the industrial production of acrylamide (more than 30,000 tons/year in the world) (*1*). However, the reaction mechanism has not been studied in detail.

NHase consists of α and β subunits with M_rs of about 23 k (*1*). The enzyme has non-heme iron (Fe-type NHase) (*2*) or non-corrinoid cobalt (Co-type NHase) (*3*) atoms as the catalytic center. Since both types of NHases exhibited very similar X-ray absorption spectra, their ligand environments were thought to be the same (*3*). In the crystal structure of Fe-type NHase from *Rhodococcus* sp. R312 (*4*), the iron center is located in a cavity at the subunit-subunit interface. The iron center is composed of four amino acid residues from the α subunit (αCys109, αCys112, αSer113, and αCys114), which are conserved in both Fe-type and Co-type NHases.

Some Fe-type NHases show an extraordinary characteristic: they lose all their catalytic activity on aerobic incubation of microorganisms in the dark (inactive state), but immediately recover it upon light irradiation (active state) (*5*). We revealed that the iron center in the inactive state was persistently associated with an endogenous nitric oxide (NO) molecule produced by microorganisms, and the photo-dissociation of the NO molecule induced the activation of NHase (*6-8*). Although the physiological meaning of this characteristic is unknown, this photoreactivity is likely to be common among Fe-type NHases (*9*).

Recently, we revealed the crystal structure of the photoreactive NHase from *Rhodococcus* sp. N-771 in the inactive state at 1.7 Å resolution (*10*). The folding pattern of the nitrosylated inactive NHase was almost the same as that of the active NHase (*4*). The atoms coordinating to the iron in the nitrosylated state were also identical to those in the photoactivated state except for the nitrogen of NO. The NO

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Abbreviations: bp, base pair(s); FITC, fluorescein isothiocyanate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; NHase, nitrile hydratase; NK24, peptide isolated from the α subunit of the NHase (αAsn[105] to αLys[128]); NO, nitric oxide; PCR, polymerase chain reaction; S/D sequence, Shine-Dalgarno sequence; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

occupied the sixth site which is solely accessible to the solvent and thought to be the catalytic site. Interestingly, the sulfur atoms of αCys112 and αCys114 were post-translationally modified to a cysteine-sulfinic acid and a cysteine-sulfenic acid, respectively (*10*). Together with another oxygen atom of αSer113, these modifications formed a claw setting of oxygen atoms capturing an NO molecule. Both modifications were confirmed by mass spectrometry, and were even conserved in the photoactivated state (*11*). This unprecedented structure is likely to enable the photoregulation of the NHase. Although there is no direct evidence, this structure is also likely to be important for the catalytic activity because it is located at the catalytic site.

To determine the functional role and the mechanism of the biogenesis of the post-translational modifications, characterization of a recombinant NHase produced in *E. coli* and site-directed mutagenesis are indispensable. However, attempts to overproduce Fe-type NHases from various sources in *E. coli* have been unsuccessful (*12-14*). The relative NHase activities in *E. coli* expressing NHase from *Rhodococcus* sp. N-774 and *Pseudomonas chlororaphis* B23 were around 10-25.5 and 10-50% of those of the native strains, respectively (*12-14*). The flanking sequences are known to be important for functional expression of both Fe and Co type NHases from *Rhodococcus* sp. N-774 (*13*), *P. chlororaphis* B23 (*14*), *Rhodococcus rhodochrous* J1 (*15, 16*), and *Pseudomonas putida* 5B (*17*). The orfs in the flanking regions have been thought to encode the proteins responsible for transporting Fe or Co (*16*).

We have succeeded in producing a large amount of a functional NHase in *E. coli* by optimizing the cultivation conditions and co-expressing the NHase activator encoded downstream of the β subunit gene. The thus produced NHase was purified and characterized. Mass spectrometry of the peptide obtained from the recombinant NHase showed that its αCys112 was also modified to a cysteine-sulfinic acid like in the native NHase. The recombinant NHase was also reversibly inactivated on the addition of NO.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, Enzymes, and Reagents—The *E. coli* strains used in this study were DH5α for preparation of plasmids and JM109(DE3) for expression of the NHase. The plasmids, pUC118 and pUC18, were used for cloning and sequencing of the NHase operon. The plasmid, pET23c, was used for expression of the NHase and NHase activator. The β subunit was also expressed using pET23d and the kanamycin resistant plasmid, pHSG299. The plasmid, pRCN1, encompassing the latter half of the NHase operon from *Rhodococcus* sp. N-771 was used for construction of NHase expression systems (Fig. 1) (*18*).

Restriction and modification enzymes were purchased from Takara Shuzo. Ex Taq DNA polymerase and other reagents for the polymerase chain reaction (PCR) were also obtained from Takara Shuzo. Nylon membranes, and the ECL random prime labeling and detection kit were the products of Amersham Pharmacia Biotech. Synthetic oligonucleotides were obtained from Sawady Technology. SDS-PAGE gels (8-16%) were obtained from TEFCO. All other reagents used were of the highest biochemical grade available.

DNA Manipulation—Preparation of plasmid DNA, isolation and purification of DNA fragments, DNA ligation, and gel electrophoresis were carried out according to standard techniques (*19*).

The total DNA of *Rhodococcus* sp. N-771 was partially digested with *Sau*3AI, and DNA fragments of 8-10 kb were subcloned in the *Bam*HI site of pUC118. The thus constructed DNA library was screened by colony hybridization using a *Pst*I fragment of pRCN1 encompassing the NHase α and β subunit genes and orf1188 as a probe. Labeling and detection were performed with the ECL random prime labeling and detection kit, a clone, pRCN5, with an about 9 kb insert DNA being obtained. The obtained gene was sequenced on both strands by the dideoxy chain termination method using a fluorescent DNA sequencer (ALF DNA sequencer II; Amersham Pharmacia Biotech).

PCR was performed with a Perkin-Elmer model 480 DNA thermal cycler. In 100 μl of reaction mixture, 1 ng of template DNA and 100 pmol of each primer were present. Each mixture was incubated at 94°C for 3 min, and then subjected to 30 cycles of 1 min of denaturation at 94°C, 30 s of annealing at 50°C, and 2 min of elongation at 72°C.

The plasmids for expression of NHase in *E. coli* were constructed by the following procedure. The genes for the NHase α and β subunits were amplified by PCR with a primer set (5′-AT-AGG-ATC-CAT-ATG-TCA-GTA-ACG-ATC-GAC-CAC-3′ and 5′ TG-AAGCTT-TCA-GGC-CGC-AGG-CTC-GAG-GTA 3′) using pRCN1 as a template. After sequence confirmation, the amplified fragment was subcloned between the *Nde*I and *Hin*dIII sites of pET23c (pRCN102). The orf1188 was also amplified from pRCN1 with a primer set (5′ TG-AAG-CTT-CAT-ATG-GTC-GAC-ACA-CGA-CTT-CCG 3′ and 5′-TTG-AAT-TCT-CAA-ACG-GTC-TGG-TCG-GTA-TA-3′), digested with *Nde*I and *Eco*RI, and then introduced into pET23c. The thus constructed pRCN202 was digested with *Xba*I and *Eco*RI, and the 1.4-kbp fragment containing orf1188 with an S/D sequence was introduced into pUC18 (pRCN203). Then, pRCN103 was constructed by subcloning the *Eco*RI/*Hin*dIII fragment of pRCN203 downstream of the β subunit gene in pRCN102. The plasmid, pRCN104, with an authentic sequence between the β subunit gene and orf1188, was generated by replacing the 1 kb *Nco*I fragment of pRCN-103, which encompasses the latter half of the β gene and the former half of orf1188, with that of pRCN1.

The plasmid, pRCN1, was digested with *Nco*I and *Bgl*II, and the 1 kbp fragment corresponding to the β subunit gene was subcloned into pET23d. The thus constructed pRCMβ was digested with *Bgl*II and *Eco*RI, and the 1.2 kbp fragment, containing the β subunit gene with an S/D sequence was introduced into pHSG299 to generate the plasmid, pHSGβ.

All the constructed plasmids were confirmed by DNA sequencing.

Expression and Purification of the Recombinant NHase and the NHase Activator—*E. coli* JM109(DE3) cells transformed with pRCN102, pRCN103, pRCN104, pRCN102+pHSGβ, pRCN103+pHSGβ, and pRCN104+pHSGβ were grown in 500 ml of Luria-Bertani (LB) medium containing ampicillin (150 μg/ml), or ampicillin (150 μg/ml) and kanamycin (100 μg/ml) at 37, 30, and 27°C. When the optical density at 600 nm reached 0.5, isopropyl-β-D-thio-

galactopyranoside (IPTG) (0.1 mM) and ferric citrate (2 mM) were added. The cultivation was further continued for 12 h, and the cells were harvested by centrifugation (5,000×*g*, 10 min, 4°C). The collected cells were suspended in 40 ml of 50 mM *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid (HEPES) buffer containing 44 mM *n*-butyric acid adjusted to pH 7.5 at room temperature with NaOH (HBS1 buffer), and then disrupted by sonication (20-kc tip, 40 W, 8 min). The cell debris was removed by centrifugation (15,000×*g*, 60 min, 4°C).

For purification of the recombinant NHase from JM109-(DE3)/pRCN104+pHSGβ, ammonium sulfate (1 M) was added to the supernatant, and the supernatant was applied to a butyl-Toyopearl column (2.5×25 cm) (Tosoh, Tokyo) equilibrated with HBS1 buffer containing 1 M ammonium sulfate. After the column had been washed with the equilibration buffer, the protein was eluted with a linear gradient of ammonium sulfate (1 to 0.5 M, 1 liter). Fractions exhibiting high activity were collected and dialyzed against 50 mM HEPES buffer containing 44 mM *n*-butyric acid adjusted to pH 7.0 at room temperature with NaOH (HBS2 buffer). The dialyzed samples were applied to a DEAE-Toyopearl column (1.5×25 cm) (Tosoh) equilibrated with HBS2 buffer. After the column had been washed with the HBS2 buffer containing 120 mM NaCl, the NHase was eluted with a linear gradient of NaCl (120 to 350 mM, 400 ml). The purity of the NHase was determined to be more than 95% by SDS-PAGE. The NHase was stored at 4°C as a suspension in 60% saturated ammonium sulfate.

For expression of the NHase activator, *E. coli* JM109-(DE3) transformed with pRCN202 was grown in 4 ml of LB medium at 37 and 27°C. When the optical density at 600 nm reached 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 0.1 mM, the cultivation was continued for 12 h, and then the cells were harvested by centrifugation (5,000×*g*, 10 min, 4°C).

Characterization of the Recombinant NHase—Nitrile hydratase activity was measured by following the hydration of methacrylonitrile spectrophotometrically in 0.1 M sodium phosphate, pH 7.5, at 30°C ($\varepsilon_{224}=3.4\ mM^{-1}\cdot cm^{-1}$) (*20*). One unit of activity is defined as 1 μmol of methacrylamide produced per min from methacrylonitrile. Exposure of the recombinant NHase to NO gas was performed as described previously (*7*). Preparation of the α subunit and the peptide fragment from the recombinant NHase was performed as described previously (*11*). The molecular masses of peptide fragments were determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). MALDI-TOF MS was performed with a Reflex (Bruker) equipped with a delayed extraction ion source. Samples were prepared by mixing 0.5 μl of the 10 mg/ml 2,5-dihydroxybenzoic acid matrix solution with 0.5 μl of the sample dissolved in 33% acetonitrile and 0.07% trifluoroacetic acid on the target. Positive ion mass spectra were acquired in the reflectron mode at 28.5-kV accelerating voltage. The mass scale was calibrated using commercially available peptides.

Other Methods—The concentrations of the recombinant NHase and the isolated subunit were determined with Coomassie Brilliant Blue dye reagent using bovine serum albumin as the standard (*21*). The amino acid sequence was determined with a 477A protein sequencer connected on line to a 120A PTH analyzer (Perkin-Elmer Applied Biosystems) and a 835 Amino Acid Analyzer (Hitachi), respectively. Proteins were analyzed by polyacrylamide gel electrophoresis on either 13% or 8–16% polyacrylamide gels in the presence of SDS (SDS-PAGE) (*22*). Gels were stained with Coomassie Brilliant Blue R-250. The sequences were analyzed using the software, Genetyx-MAC (Software Development) and Sequencher™ (Gene Code). Sequence alignment was performed with the program, "ClustalW."

RESULTS

Cloning and Sequencing of the Nitrile Hydratase Operon of Rhodococcus sp. N-771—It has already been reported

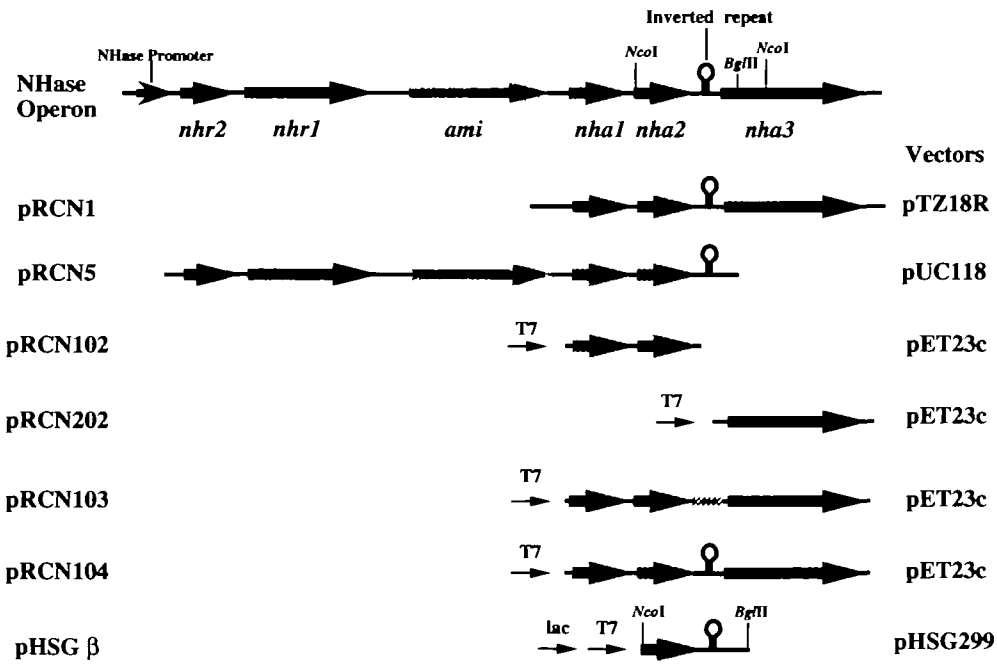

Fig. 1. **Schematic representation of the plasmids used in this study and the gene organization of the nitrile metabolic operon in *Rhodococcus* sp. N-771.** The inverted repeat between *nha2* and *nha3* is schematically shown. In pRCN103, the sequence is replaced by the multi-cloning site of the pUC vector. The details are given under "EXPERIMENTAL PROCEDURES."

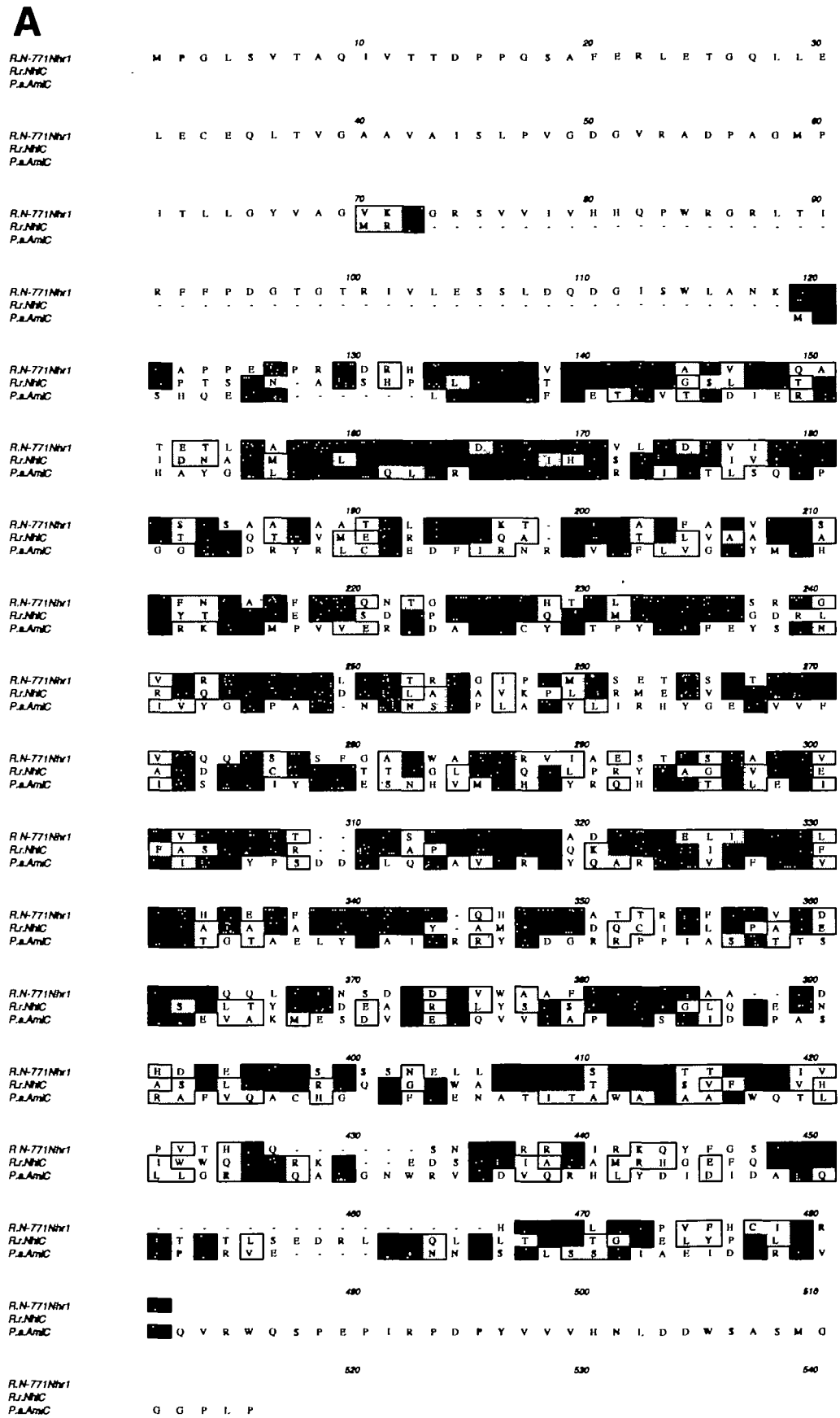

Fig. 2A

that the NHase operon of *Rhodococcus* sp. N-774 consists of at least 4 genes (*12, 13*). Three of them encode amidase, NHase α subunit and NHase β subunit. The fourth open reading frame, orf1188, encodes a polypeptide of 396 amino acids homologous to P47k, which is also encoded by a gene in the NHase operon of *Pseudomonas chlororaphis* B23 (*14*). Both the product of orf1188 and P47k were reported to be important for functional expression of NHase (*13, 14*). We also cloned a 9 kb DNA fragment encompassing the latter half of the NHase operon from *Rhodococcus* sp. N-771. The nucleotide sequences of the NHase genes and orf1188 were exactly the same as those of *Rhodococcus* sp. N-774 (*18*). The genes encoding amidase, NHase α subunit and NHase β subunit are designated as *ami, nha1*, and *nha2*, respectively (Fig. 1).

It has been reported that there are at least five open reading frames in addition to the H-NHase α- and β-subunit genes in the operon of the high molecular mass-nitrile hydratase (H-NHase) from *Rhodococcus rhodochrous* J1. Some of them encode proteins responsible for regulation of the expression of the operon. Thus, it can be easily inferred that some other proteins are also encoded in the 5′ terminal region of the NHase operon of *Rhodococcus* sp. N-771 (N-774). However, upstream of the amidase gene in *Rhodococcus* sp. N-771 (N-774) has not been sequenced, and the total operon structure and regulatory sequence of the NHase operon remain unknown.

To determine the total structure of the NHase operon from *Rhodococcus* sp. N-771, we cloned the upstream region of the NHase operon by colony hybridization using a DNA fragment containing the NHase gene from *Rhodococcus* sp. N-771 as a probe. Then, a clone, pRCN5, was isolated. The insert of pRCN5 is about 9 kb in length and encompasses the upstream region of the NHase operon. The nucleotide sequence showed that two ORFs and a putative promoter region existed in upstream of the amidase gene (Fig. 1). The nucleotide sequence of the putative promoter region showed significant similarity with the symmetrical sequence motif and promoter regions of the *hox* operon from *R. opacus* MR11 (*23*) (59.70% homology). Both the two newly found ORFs, designated as nhr1 and nhr2, start with the low frequency initiation codon, GTG. The initiation codons were preceded by Shine-Dalgarno (S/D) sequences at reasonable distances. The first ORF, *nhr1*, encodes a protein of 454 amino acids exhibiting a significant similarity with NhlC from *R. rhodochrous* J1 (30.89% identity) (*24*) and AmiC from *P. aeruginosa* (19.78% identity) (*25*). The second ORF, named *nhr2*, is 366 nucleotides long, and should encode a protein of 122 amino acids, and shows significant similarity in amino acid sequence with *nhlD* from *R. rhodochrous* J1 (32.21% identity) (Fig. 2) (*24*).

Although downstream of orf1188 of pRCN1 was sequenced, no open reading frame was found. Thus, the six ORFs in the nitrile metabolism gene clusters, named the NHase operon, are probably translated in a polycistronic manner.

Overproduction of the NHase in E. coli—To express active NHase in *E. coli*, we constructed various NHase expression plasmids using a T7 expression system, and transformed *E. coli* JM109 (DE3) with the resulting plasmids (Fig. 1). In the plasmid, pRCN102, only the α and β subunit genes were subcloned downstream of the T7 promoter. The plasmids, pRCN103 and pRCN104, contain orf1188 in addition to the α and β subunit genes. The sequence between the β subunit gene and orf1188 in pRCN104 was the same as the original sequence. In pRCN103, the inverted repeat between the β subunit gene and orf1188 was replaced by the multi-cloning site of the

TABLE I. **NHase activity in cell-free extracts of *E. coli* cells harboring various expression plasmids.** *E. coli* transformants were cultured at 37, 30, and 27°C. The NHase activities of the purified NHase and recombinant NHase were 1,732.9 and 1,635.0 units/mg, respectively. The details are given under "EXPERIMENTAL PROCEDURES." [a]ND, not detected.

	NHase activity (units/mg)		
	37°C	30°C	27°C
pRCN102	ND[a]	ND	—
pRCN103	ND	49.6	—
pRCN104	ND	33.8	—
pRCN102+pHSGβ	ND	ND	ND
pRCN103+pHSGβ	ND	184.6	411.8
pRCN104+pHSGβ	ND	117.2	452.2

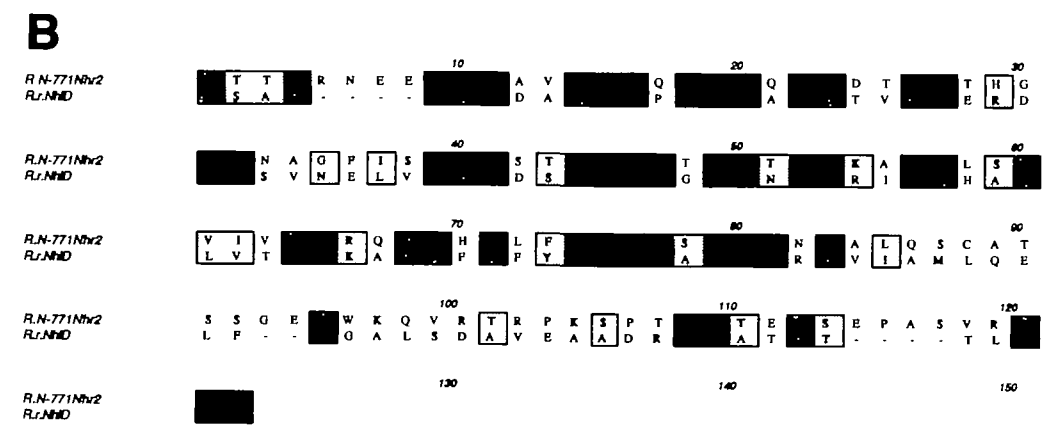

Fig. 2. **Alignment of the amino acid sequences of NHase regulators.** Conserved amino acid residues are indicated by shaded boxes. Homologous amino acids are boxed. A: Alignment of NHase regulator 1 from *Rhodococcus* sp. N-771 (*R.* N-771 Nhr1) with NhlC from *R. rhodochrous* J1 (*R.r.* NhlC) and AmiC from *P. aeruginosa* (*P.a.* AmiC). B: Alignment of NHase regulator 2 from *Rhodococcus* sp. N-771 (*R.* N-771 Nhr2) with NhlD from *R. rhodochrous* J1 (*R.r.* NhlD).

pUC vector. *E. coli* cells harboring these plasmids were grown at 37°C, and expression was induced by the addition of IPTG at the logarithmic phase. SDS PAGE analysis showed that all *E. coli* cells harboring pRCN102, pRCN103, and pRCN104 expressed large amounts of NHase (data not shown). However, all formed inclusion bodies, and the crude extracts showed no NHase activity (Table I).

Then, the transformants were grown at 30°C, the temperature at which *Rhodococcus* sp. N-771 normally grows. At this temperature, the crude extracts of JM109(DE3)/pRCN104 and JM109(DE3)/pRCN103 showed NHase activity (Table I). In contrast, there was almost no NHase activity in the crude extract of JM109(DE3)/pRCN102. The amounts of NHase produced in the transformants were examined by SDS-PAGE (Fig. 3). In all transformants, the amount of NHase reached about 50% of the total protein, and most of it was in the insoluble fraction. The amounts of the α and β subunits expressed were not equal. The level of expression of the β subunit was fairly low compared with that of the α subunit. The transformant harboring pRCN-103 expressed a protein of about 47 kDa in addition to NHase (Fig. 3). The N-terminal peptide sequence showed that the protein was the product of orf1188. Since the expression of the protein was inhibited in JM109(DE3)/pRCN104, the inverted repeat between the β gene and orf1188 is likely to function as a terminator in *E. coli*. *Rhodococcus* sp. N-771 expresses a rather large amount of nitrile hydratase. However, the expression level of orf1188 is not so high (data not shown). Thus, even in *Rhodococcus* sp. N-771, the inverted repeat functions like a terminator, and the expression of orf1188 is down-regulated.

Although some of the expressed NHase correctly folded into the active form in JM109(DE3)/pRCN104 and JM109(DE3)/pRCN103 at 30°C, most folded incorrectly and formed inclusion bodies even under these conditions. SDS-PAGE showed that the inclusion bodies are mainly composed of the α subunit similar to those obtained at 37°C (data not shown). This suggests that the incorrect folding of the NHase is partly due to a shortage of the β subunit. Then, we attempted to increase the β subunit production by transforming *E. coli* harboring NHase expression plasmids with the plasmid, pHSGβ. The NHase activities in crude extracts of these transformants were also examined (Table I). At 37°C, no transformants showed NHase activity and the expressed polypeptides accumulated as inclusion bodies. Then, these transformants were cultured at a lower temperature. At 30°C, the *E. coli* transformants harboring pRCN103+pHSGβ and pRCN104+pHSGβ showed fairly higher specific NHase activity than that without pHSGβ (Table I). SDS-PAGE showed that relatively equivalent expression of the α and β subunits was obtained in all transformants (Fig. 3).

At 27°C, the *E. coli* transformants harboring pRCN104+pHSGβ and pRCN103+pHSGβ both exhibited fairly high specific NHase activity. In particular, the specific activity of JM109(DE3)/pRCN104+pHSGβ reached 452.2 units/mg protein. The amount of produced NHase was calculated to be about 25% of the total soluble protein (Table I). However, JM109(DE3)/pRCN102+pHSGβ was still inactive even at the lower temperature.

These results clearly demonstrate that the protein encoded by orf1188 is required for the functional expression of NHase. Thus, we named the protein, NHase activator. Then, orf1188 was designated as *nha3*. Expression of the NHase activator is down-regulated by a terminator and overexpression of the NHase activator did not affect the NHase activity (Table I and Fig. 3). Thus, it is suggested that the NHase activator does not form a stoichiometric complex with NHase, but catalytically activates the NHase.

The NHase Activator Is a Soluble Protein Which Can Not Fold Correctly at 37°C—We have demonstrated that functional expression of NHase depends on expression of the NHase activator and *E. coli* harboring expression plasmids must be grown at lower temperatures of 30 to 27°C. We assumed that the temperature dependency might not be related to the NHase itself, but to the NHase activator. Then, JM109(DE3) harboring pRCN202 was grown at 37 and 27°C. Although the NHase activator was markedly accumulated in *E. coli* cells at 37°C, the accumulation occurred in the insoluble fraction similar to in the case of JM109(DE3)/pRCN103 (data not shown). But, when JM109(DE3)/pRCN202 was grown at 27°C, the expressed NHase activator was mainly found in the soluble fraction (Fig. 4). This shows that the NHase activator is a soluble protein, but that it can not fold correctly at 37°C. Therefore, the incorrect folding of the NHase at 37°C might be due to the inactive expression of the NHase activator. The NHase activity of the recombinant *E. coli* cells grown at 30°C varied a lot with slight changes in the culture conditions. This could be attributed to the instability of the NHase activator at 30°C.

Characterization of the Recombinant NHase—The recombinant NHase was purified by almost the same procedure as that used for the native enzyme (*26*). The final preparation of the recombinant NHase was almost homogeneous on SDS-PAGE (data not shown). The purified recom-

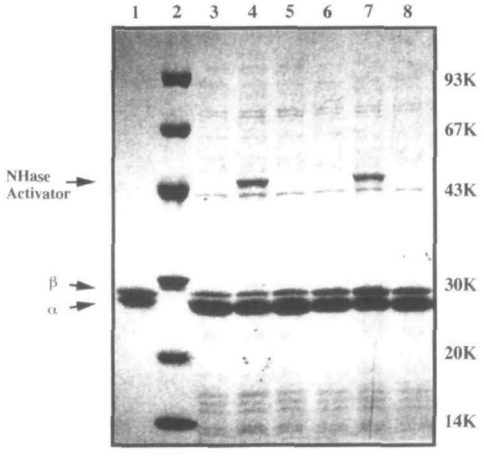

Fig. 3. **Expression of the NHase and NHase activator in *E. coli*.** *E. coli* cells harboring expression plasmids were grown at 30°C. The cell suspensions (5 O.D./ml, 0.01 ml) in HBS1 buffer were precipitated with 0.01 ml of trichloroacetic acid (30% [w/v]). The precipitates were dissolved in the sample buffer for SDS-PAGE. About 10 μg of total protein was loaded on each lane of a 8-16% SDS-polyacrylamide gel. After SDS-PAGE, protein was stained with Coomassie Brilliant Blue R-250. Purified native NHase, lane 1; molecular weight markers, lane 2; JM109(DE3)/pRCN102, lane 3; JM109(DE3)/pRCN103, lane 4; JM109(DE3)/pRCN104, lane 5; JM109(DE3)/pRCN102+pHSGβ, lane 6; JM109(DE3)/pRCN103+pHSGβ, lane 7; JM109(DE3)/pRCN104+pHSGβ, lane 8.

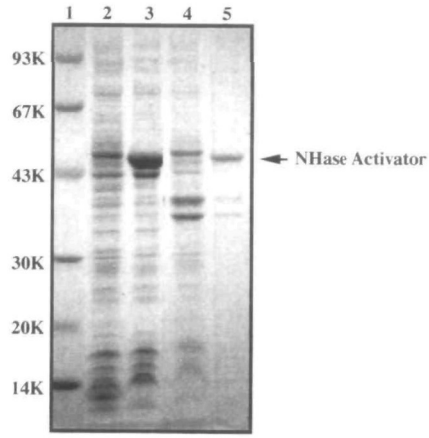

Fig. 4. **Expression of the NHase activator in the soluble protein at 27°C.** *E. coli* cells were grown at 27°C and then harvested by centrifugation. The cells were suspended in 0.8 ml of HBS1 buffer and then disrupted by sonication. The sonicates were centrifuged at 15,000×*g* for 10 min. The resulting supernatants were used as soluble fractions, and the pellets were resuspended in 0.8 ml of HBS1 buffer and then used as insoluble fractions. Ten micrograms of total soluble protein or insoluble protein was loaded on each lane of 8-16% SDS-polyacrylamide gel. After SDS-PAGE, protein was stained with Coomassie Brilliant Blue R-250. Molecular weight markers, lane 1; soluble protein of JM109(DE3)/pET23c, lane 2; soluble protein of JM109(DE3)/pRCN202, lane 3; insoluble protein of JM109(DE3)/pET23c, lane 4; insoluble protein of JM109(DE3)/pRCN202, lane 5.

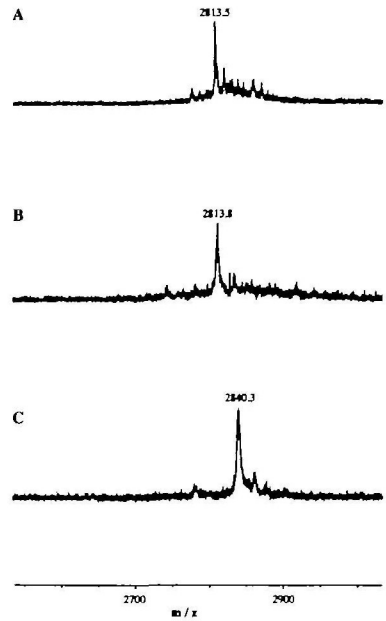

Fig. 5. **MALDI-TOF MS spectra of the tryptic peptide, NK24, after carboxymethylation and reduction.** A: NK24 obtained from the recombinant NHase prepared from JM109(DE3)/pRCN104+pHSGβ; B: NK24 from the native NHase; C: NK24 from the recombinant α subunit which was expressed as inclusion bodies. The masses of the NK24 with two and three carboxymethyl groups were calculated to be 2,779.3 and 2,837.3, respectively. The details are given under "EXPERIMENTAL PROCEDURES."

binant NHase showed almost the same enzymatic activity (1,635.0 units/mg, K_m value, 2.0 mM) as the native enzyme (1,732.9 units/mg, K_m value, 2.0 mM) and gave almost the same UV-VIS absorption spectra (in the active state) (*27*) (data not shown).

To determine whether or not αCys112 of the recombinant NHase is also post-translationally modified to a cysteine-sulfinic acid (*11*), we prepared a peptide, NK24 (Asn105-Lys128), of the α-subunit and determined the molecular mass of the peptide by MALDI-TOF mass spectrometry. The molecular mass of NK24 was 2,697.7 m/z, which is 33 Da larger than that deduced from the corresponding gene sequence (data not shown). Then, we carboxymethylated the Cys residues after reduction. Cys109 and Cys114 were identified as carboxymethylcysteines, whereas Cys112 remained undetectable. The molecular mass of reduced and carboxymethylated NK24 was determined to be 2,813.5 by MALDI-TOF MS (Fig. 5A). This value is almost the same as that for the native NHase (2,813.8) (Fig. 5B), and is 34 Da larger than the expected value (2,779.3). In contrast, the NK24 prepared from the incorrectly folded α subunit exhibited no post-translational modification, and all three cysteines were carboxymethylated as observed in the previous study (Fig. 5C) (*11*). Hence, αCys112 of the recombinant NHase expressed in *E. coli* is post-translationally modified to cysteine-sulfinic acid by oxygen like for the native NHase only when the non-heme iron center is correctly formed.

Then, we investigated the effect of NO on the recombinant NHase. Exposure of the recombinant NHase to NO resulted in the complete disappearance of the activity (data not shown). However, 44.6% of the activity of the recombinant NHase was restored after light irradiation, indicating that the NHase inactivated by NO exposure can be photoactivated like the native NHase (*7*). The low recovery ratio is likely to be due to the irreversible inactivation caused by excess NO. The photoinduced difference spectrum of the recombinant NHase exposed to NO (after minus before light irradiation) exhibiting a negative peak at 370 nm and a positive peak at 710 nm (data not shown) was essentially identical to that of the native NHase (*7*).

DISCUSSION

We have revealed the structure of the NHase operon in *Rhodococcus* sp. N-771. We identified new two ORFs (*nhr1* and *nhr2*) in the upstream region. The amino acid sequences deduced from *nhr1* and *nhr2* exhibit marked similarities to regulators, NhlC and NhlD of the *R. rhodochrous* J1 L-NHase, respectively (*24*). The *R. rhodochrous* J1 L-NHase is induced by various amide compounds, and NhlC and NhlD are responsible for the induction by amides. However, NHase production in the *Rhodococcus* sp. N-771 was not induced by amide compounds (*28*). Thus, *nhr1* and *nhr2* in *Rhodococcus* sp. N-771 might have lost their functions.

In the expression vectors, pRCN102, pRCN103, and pRCN104, the initiation codon of the α subunit gene subcloned is at the *Nde*I site of pET23c. Thus, in these vectors, the α subunit gene is preceded by an S/D sequence with an appropriate interval sequence, which is optimal for expression in *E. coli*. In contrast, the β subunit is expressed using the original ribosomal binding sequence. The ribosomal binding site of the β subunit is almost the same as the ideal S/D sequence of *E. coli*, but the interval is rather short. Therefore, the relatively low expression of the β

subunit is likely to be due to low initiation of translation.

When *E. coli* cells harboring NHase expression plasmids were co-transformed with pHSGβ, the expression levels of the α and β subunits varied a lot. This is probably due to changes in the copy numbers of the plasmids with the slight changes in the culture conditions.

The NHase activator shows significant similarity in the amino acid sequence with the iron transporter gene of magA from *Magnetospirillum* sp. AMB-1 (*29*), and the cobalt transporter gene of *nhlF* from *R. rhodochrous* J1 (*16*). *Magnetospirillum* sp. AMB-1 is a magnetic bacterium, and its proposed magnetic particle synthesis system (*29*) involves (i) uptake of iron; (ii) transport of iron to the cytoplasmic space and across the magnetic particle membrane; (iii) precipitation of hydrated ferric oxide within vesicles; and (iv) phase transformation of the amorphous iron phase to magnetite, during both nucleation and surface-controlled growth (*29*). The *magA* gene product is thought to be an ATP-dependent iron transporter (*29*). The NHase activator contains both of the two consensus motifs in the ATP-binding protein (*13*). The product of *nhlF*, NhlF, shows significant sequence similarity with that of *hox*N from *Alcaligenes eutrophus*, which is considered to be involved in nickel uptake. Sequence and hydropathy plot analyses have shown that NhlF encodes a 352-aa protein with eight hydrophobic putative membrane-spanning domains. NhlF expression in *R. rhodochrous* ATCC 12674 and *E. coli* JM109 allows the uptake of ^{57}Co in their cells, but not of ^{63}Ni. These results suggest that the NHase activator is also a membrane-bound Fe transporter. However, our result contradicts this assumption. The NHase activator is not a membrane-bound protein but a soluble protein. Expression of the Co-type NHase from *P. putida* 5B in *E. coli* also requires co-expression of a novel downstream gene encoding a protein (P14K) of 127 amino acids. P14K is not a membrane protein, but a soluble protein too. Cobalt must be present during protein production for maximal activity of this NHase. In contrast, no activity is recovered when cobalt is added to the purified NHase prepared from cells grown in cobalt-free medium. However, NHase activity is recovered by incubation with P14K in the presence of cobalt. This suggests that P14k is involved in the incorporation of a cobalt ion into the NHase. The NHase activator might also be required for the incorporation of Fe iron in the NHase. There is another possibility, *i.e.* that these proteins might be required for modification of the cysteine residues in the catalytic center if they are important for the catalysis.

Previously, we reported that αCys112 of a recombinant α subunit, which was expressed as inclusion bodies, was not modified to a cysteine-sulfinic acid. However, the present study showed that this cysteine residue of the functionally expressed recombinant NHase was post-translationally modified to a cysteine-sulfinic acid (Fig. 5) like for the native NHase from *Rhodococcus* sp. N-771. Since the isolated peptide was unstable, the modification of αCys114 was not confirmed. However, the fact that αCys109 and αCys114 in the peptide easily formed a disulfide bond suggests that αCys114 is also modified to a cysteine-sulfenic acid. Moreover, the recombinant NHase was also changed to the inactive form by NO similar to the native NHase (*7*). It is suggested that the claw setting formed by three oxygens of cysteine-sulfinate, cysteine-sulfenate, and αSer113 is important for the interaction with NO (*10*). This also supports that αCys114 of the recombinant NHase is modified to a cysteine-sulfenic acid.

The mechanism underlying the biogenesis of these post-translational modifications remains unknown. There are several possibilities for the mechanism of this oxygenation in the iron center. As suggested for glutathione peroxidase (*30, 31*), NO or some higher oxides of nitrogen including $ONOO^-$ may contribute to the thiol oxidations, which have been found in human serum albumin (*32-34*). However, this mechanism is excluded by the fact that αCys112 in the recombinant NHase expressed in *E. coli* was also modified to a cysteine-sulfinic acid. Although enzymatic oxidation by the NHase activator might be possible, it showed no sequence homology with cysteine dioxygenases (*35, 36*). Therefore, it is most plausible that the modifications comprise oxygenation, occurring in a self processing manner to form a non-heme iron center, similar to for the formation of the topa quinone (TPQ) cofactor in bacterial monoamine oxidases (*37-40*). A mechanism for the biosynthesis of TPQ has been proposed by Cai and Klinman (*40*). The reaction begins with (i) copper binding to O_2 and reduction to a hydroperoxide. The activated oxygen then (ii) hydroxylates the tyrosine ring converting it to dopa, which (iii) is oxidized to the topa quinone by a second molecule of O_2 (*40*). It is probable that the αCys112 in the iron center is modified through a similar process to that described above.

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