# Motif CXCC in nitrile hydratase activator is critical for NHase biogenesis in vivo

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**Abstract Nitrile hydratase (NHase) activator from *Rhodococcus* sp. N-771 is required for NHase functional expression. The motif 73CXCC76 in the NHase activator sequence was here revealed to be vital for its function by site-directed mutagenesis. All three substitutions of the cysteines by serines resulted in a much lower level of expression of active NHase. Furthermore, interaction between NHase activator and NHase was detected and the critical role of NHase activator was not exhibited in the cysteine oxidization process of NHase. These findings suggest NHase activator mainly participates in iron trafficking in NHase biogenesis as an iron type metallochaperone.**


*Key words:* Nitrile hydratase; Nitrile hydratase activator; Site-directed mutagenesis; Protein–protein interaction; Iron trafficking

## 1. Introduction

Nitrile hydratase (NHase) is composed of α and β subunits, contains either a non-heme iron(III) or non-corrinoid cobalt(III), and catalyzes the hydration of various nitriles to the corresponding amides [1–5]. NHase from *Rhodococcus* sp. N-771 is a photoactive metalloenzyme and in essence its activity is regulated by the conversion of nitrosylation and denitrosylation of the iron active center. NO dissociation can change inactive NHase into the active form, whereas nitrosylation can inactivate NHase [6–9]. Recently, crystal structure analysis at 1.7 Å resolution has revealed that the iron in inactive NHase is associated with three cysteine residues in the α subunit, αCys109 and two post-translationally oxidized cysteines, α cysteine sulfinic acid 112 and α cysteine sulfenic acid 114 [10]. In addition, the NHase precursor (recombinant NHase without cysteine oxidization) does not exhibit catalytic activity [11], suggesting that cysteine oxidization is required for NHase to show its function.

Our previous results have also demonstrated that the NHase operon consists of *nhr2*, *nhr1*, *ami*, *nha1*, *nha2*, and *nha3*, six genes encoding NHase regulator 2, NHase regulator 1, amidase, NHase α subunit, NHase β subunit and NHase activator, respectively. The soluble protein NHase activator, encoded by the open reading frame *nha3*, is essential for the functional expression of NHase in vivo [12]. The requirements of downstream protein for efficient NHase expression have been observed in other bacterial species [13–15]. However, molecular level evidence to demonstrate the role of NHase activator in the biosynthesis of NHase is still lacking.

Cysteine-rich motifs in proteins play important roles in the processes of protein–protein association, metal binding and transport, and redox reaction regulation [16–20]. Copper trafficking in vivo is known to be undertaken by copper chaperones, a new family of soluble cytoplasmic proteins [16]. In eukaryotic cells at least three copper chaperones with cysteine-rich motifs are involved in the copper trafficking pathways independently [17]. COX 17 transporting the copper to mitochondrial cytochrome oxidase possesses the CCXC metal binding motif [21,22]; Atx 1 delivering copper to CCC2 has the MTCXXC motif [23–25]; CCS mediating the copper transport process to cytosolic SOD 1 employs the motifs CXXC and CXC [26–28].

Sequence alignments of NHase activator show the members contain a highly conserved cysteine-rich motif CXCC (Table 1). Here we have performed mutagenesis in this motif to deeper understand the role of NHase activator. The substitution of each of the cysteine residues with a serine residue in the motif reduced the efficient expression of NHase dramatically. The evidence sheds light on the fact that NHase activator plays its role with specific protein binding sites during the NHase maturation process.

## 2. Materials and methods

### 2.1. Plasmid construction

Plasmid pRCN 102, pET-23c vector (Novagen, ampicillin resistance) carrying the *Nha1* and *Nha2* genes, was employed to express NHase α and β subunits [12]. To construct the plasmid for expressing carboxy-terminal $(His)_6$-tagged NHase activator, the encoding sequence was amplified by polymerase chain reaction (PCR) using pRCN 202 as template, pET-23c vector carrying the *Nha3* gene. The sense primer was 5′-TGCCGGTTCATATGGTCGACACACGACTTCCG-3′, containing a *Nde*I restriction site; the antisense primer was 5′-CGAAGCTTAACGGTCTGGTCGGTATACCCGGC-3′, containing a *Hin*dIII restriction site. Then the PCR products were purified according to the instructions of the MagExtractor DNA

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*Abbreviations:* NHase, nitrile hydratase

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fragment purification kit (Toyobo). The purified PCR products were excised with *Nde*I and *Hin*dIII and subcloned into pET-30b (Novagen, kanamycin resistance) to create the plasmid pACTHistag. To create a plasmid pACT for expressing NHase activator without $(His)_6$ tag fused, PCR was employed to amplify the encoding sequence, using pRCN 202 as template, T7 Promoter primer and T7 Terminator primer as primers. The encoding sequence was also excised with *Nde*I and *Hin*dIII and subcloned into pET-30b. The mutant pACTHistag plasmids for expressing mutant $(His)_6$-tagged NHase activators were obtained via site-directed mutagenesis with QuikChange (Stratagene) according to the manufacturer's protocol, using pACTHistag as template. The primer pairs, 5′-CTCTCGCAGAGTGCAGCAGATGGAGCCGTTTGTCATCTC-3′ and 5′-GAGATGACAAACGGCTCCATCTGCTGCACTCTGCGAGAG-3′, 5′-CTCTCGCAGAGTGCAGGAGATGCAGCCGTTTGTCATCTC-3′ and 5′-GAGATGACAAACGGCTGCATCTCCTGCACTCTGCGAGAG-3′, 5′-CTCTCGCAGAGTGGAGCAGATGCAGCCGTTTGTCATCTC-3′ and 5′-GAGATGACAAACGGCTGCATCTGCTCCACTCTGCGAGAG-3′, were used to construct the mutant pACTHistag plasmids for expressing NHase activators C73S, C75S, and C76S, respectively. The DNA sequences of all constructed plasmids were validated by sequence determination with an ABI Prism 377XL DNA sequence analyzer (Applied Biosystems).

### 2.2. Protein overexpression and NHase activity assay

Plasmid pRCN 102 and plasmid pACTHistag/mutated pACTHistag/pACT were co-transformed into *Escherichia coli* BL21(DE3)pLysS (Promega). The transformed *E. coli* strains were grown in LB medium containing 100 μg/ml ampicillin and 100 μg/ml kanamycin at 27°C with shaking at 250 rpm. When the value of $OD_{600}$ reached 0.6–0.8, protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactose (IPTG) alone or together with 0.2 mM ferric citrate. After growth at 27°C for 12 h, cells were harvested by centrifugation at 5000 rpm for 15 min at 4°C.

For NHase activity assay, the collected cells were resuspended in buffer A (50 mM Tris–HCl, pH 7.5, 40 mM *n*-butyric acid) and lysed by three cycles of freezing and thawing. Then the cell lysate was centrifuged at 15 000 rpm for 30 min at 4°C and the supernatant was subjected to NHase activity assay [29]. Experiments were repeated independently at least three times in triplicate assays. The precipitate was dissolved in buffer B (50 mM Tris–HCl, pH 7.5, 8 M urea, 30 mM dithiothreitol (DTT)) and applied for NHase reconstitution. The NHase activities of the cell extracts from the transformants expressing $(His)_6$-tagged NHase activator and NHase activator exhibited no significant difference, suggesting the fused $(His)_6$ tag at the carboxy-terminal did not alter the property to assist NHase functional expression. Thus, in the experiments to investigate the co-expression and interaction of NHase with NHase activator, $(His)_6$-tagged NHase activator was also denoted as NHase activator. Protein concentration was measured by the Bradford method (Bio-Rad).

For the immunoblotting experiment, the collected cells were resuspended in buffer C (50 mM Tris–HCl, pH 7.5, 10 mM mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 3 μg/ml aprotinin) and were lysed by three cycles of freezing and thawing. After centrifugation (15 000 rpm, 30 min, 4°C), aliquots of the cell extracts were subjected to 8–16% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to nitrocellulose membrane for blotting.

### 2.3. Purification of NHase activator and recombinant NHase α subunit and β subunit

The recombinant NHase α and β subunit proteins were produced from *E. coli* JM109(DE3) harboring the vector pET 3c or pHSG 299 containing the gene to express NHase α or β subunit, respectively. NHase α and β subunit expression and isolation have been described previously [11,32].

*E. coli* BL21(DE3)pLysS harboring plasmid pRCN 202 was used to express NHase activator as described in Section 2.2. The cell extract containing soluble NHase activator was applied to a Super Q Toyopearl column. NHase activator was eluted using a gradient of 50 mM Tris–HCl, pH 7.5, 1 mM DTT, 1 mM EDTA to 50 mM Tris–HCl, pH 7.5, 1 mM DTT, 1 mM EDTA with 1.0 M NaCl. The fraction containing NHase activator was eluted at approximately 0.56 M NaCl and precipitated with 30% saturated ammonium sulfate. The precipitate was dissolved in 50 mM Tris–HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 20% ammonium sulfate and then loaded onto a hydrophobic column equilibrated with 50 mM Tris–HCl, pH 7.5, 2 mM mercaptoethanol, 20% saturated ammonium sulfate. The column was eluted with a 20–0% saturated ammonium sulfate gradient. The fractions containing NHase activator eluted at approximately 5% saturated ammonium sulfate were pooled and dialyzed with 50 mM Tris–HCl, pH 7.5, 2 mM mercaptoethanol overnight. The purified protein was estimated to be 95% pure by SDS–PAGE and verified to be NHase activator by amino acid sequence determination.

### 2.4. Reconstitution of NHase from inclusion body

NHase was reconstituted from the α and β subunits in inclusion body based on a previously described method [11]. Briefly, inclusion body was dissolved in buffer B and incubated at 37°C for 1 h. After addition of 10 μM ferric citrate, the mixture was dialyzed at 4°C against buffer D (50 mM Tris–HCl, pH 7.5, 40 mM butyric acid, 2 mM mercaptoethanol, 10 μM ferric citrate) for 24 h under argon. Finally, the mixture was exposed under air and aliquots of the solution were withdrawn for NHase activity assay. Experiments were repeated independently at least three times in triplicate assays.

### 2.5. Ni-NTA affinity purification

The cells were expressed as described in Section 2.2 except the incubation under 27°C for 2 h after the addition of IPTG and iron. The collected cells were resuspended in equilibrium buffer (50 mM Tris–HCl, pH 7.5, 500 mM NaCl, 10 mM mercaptoethanol, 10 mM imidazole) and lysed by three cycles of freezing and thawing. After centrifugation (15 000 rpm, 30 min, 4°C), the supernatant was subjected to a Ni-NTA agarose affinity column which was pre-equilibrated with the equilibrium buffer. Then the column was washed with washing buffer (50 mM Tris–HCl, pH 7.5, 500 mM NaCl, 10 mM mercaptoethanol, 50 mM imidazole). The proteins bound with $Ni^{2+}$ by polyhistidine were eluted with eluting buffer (50 mM Tris–HCl, 300 mM NaCl, 250 mM imidazole, pH 7.5). The eluted sample was dialyzed against buffer E (50 mM Tris–HCl, pH 7.5, 10 mM mercaptoethanol) at 4°C and then aliquots of the protein samples were analyzed by immunoblotting.

### 2.6. Immunoblotting analysis

The protein samples were separated on 8–16% SDS–PAGE gels and blotted on a nitrocellulose membrane. The blotted membrane was blocked with TTBS buffer (20 mM Tris–HCl, pH 7.6, 200 mM NaCl, 0.1% Tween 20, 3% bovine serum albumin (BSA)) at 4°C. Then the blots were incubated with the primary antibodies for 1 h, with the anti-polyhistidine His probe (H-15) (Santa Cruz Biotech), 1:1000; with antiserum raised against NHase α subunit or β subunit, 1:5000. After washing with TTBS, the blots were incubated with anti-rabbit horseradish peroxidase-labeled secondary antibody, 1:10000. Detection was performed according to the instructions with the ECL plus kit (Amersham Pharmacia Biotech).

### 2.7. Cysteine oxidization of NHase precursor

NHase precursor was reconstituted by purified recombinant α and β subunits as described in Section 2.4. The dialysis time was enough for recombinant α and β subunits to assemble into NHase precursor sufficiently in our experiments by NHase activity and high performance liquid chromatography detection. Then, the mixture containing NHase precursor was incubated with equimolecular purified NHase activator for 8 h under argon. Finally, the mixture was exposed under air and an aliquot of solution was withdrawn for NHase activity assay, BSA was used as a control protein.

## 3. Results

### 3.1. Site-directed mutagenesis of NHase activator

Three conserved cysteines in positions 73, 75 and 76 of NHase activator were replaced by serines to investigate the role of the biosynthesis of NHase in vivo. All the substitutions in NHase activator (C73S, C75S and C76S) led to a dramatic reduction of NHase activity of cell extract when NHase and NHase activator/mutated NHase activators were co-expressed in *E. coli* BL21(DE3)pLysS (Fig. 1). When the transformants were grown in iron-abundant medium, NHase activity of the

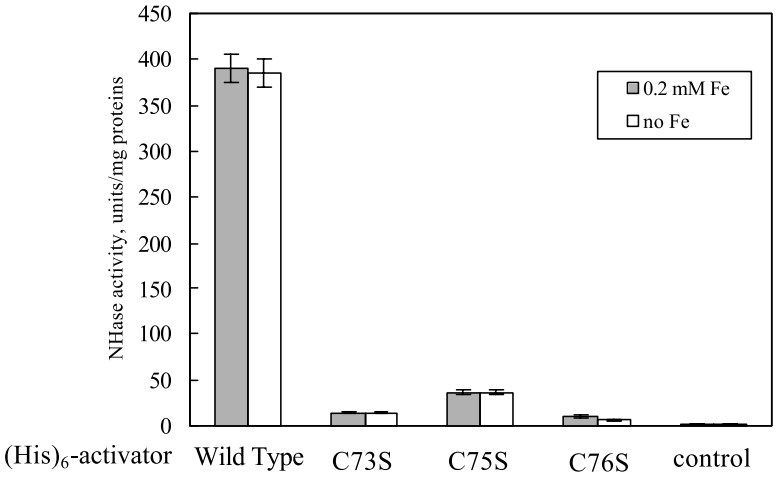


Fig. 1. Co-expression of NHase and NHase activator/mutant NHase activator in *E. coli*. Plasmids pRCN 102 and pACTHistag/mutant pACTHistag were co-transformed into *E. coli* BL21(DE3)pLysS. The transformants were grown at 27°C and induced with 0.1 mM IPTG alone or together with 0.2 mM ferric citrate for 12 h. The collected cells were resuspended in buffer 50 mM Tris–HCl, pH 7.5, 40 mM *n*-butyric acid and lysed by freezing and thawing. The cell lysate was centrifuged and the supernatant was picked up for the NHase activity assay. In the control, only pRCN 102 was transformed into *E. coli* BL21(DE3)pLysS.

cell extract from the transformant expressing wild NHase activator got to 390 ± 15 U/mg protein, NHase activity of cell extract from transformants expressing mutated NHase activator C73S, C75S and C76S was 14 ± 1.2, 36 ± 2.5, 10 ± 1.1 U/mg protein, respectively. The yields of efficient NHase from transformants expressing mutant NHase activators were much less than 10% of wild type NHase activator. In control experiment, little NHase activity was detected in the cell extract from the transformant expressing NHase alone. Therefore, the CXCC motif in the NHase activator is critical for the functional expression of NHase in vivo.

### 3.2. Requirement of NHase activator for NHase functional expression even in metal-rich medium

NHase activator exhibits a similar behavior as copper chaperones to assist the target protein to get the functional conformation with a similar cysteine-rich motif. However, some copper chaperones show their functions under copper-limited conditions, in copper-rich medium the target proteins can form into their active conformation in the absence of copper chaperones [30]. But even in iron-rich medium NHase activator was required for the functional expression of NHase in vivo. The active NHase yields are independent of additional iron in LB medium, only dependent on the transformant strain (Fig. 1). In the transformants expressing the mutant NHase activators, even when the LB medium iron concentration reached the toxic level (10 mM ferric citrate) only a little functional NHase was produced, like as in sole LB medium (data not shown). In a control experiment where NHase was expressed alone, a similar result was obtained [31].

### 3.3. Immunoblotting analysis for the co-expression of NHase and NHase activator

Immunoblotting was used to examine the cell extract from *E. coli* in which NHase and NHase activator/mutated NHase activators were co-expressed (Fig. 2). NHase activator or mutated NHase activators in the cell extract represented by polyhistidine were detected to be expressed at the same level, suggesting the site mutations (C73S, C75S and C76S) do not significantly affect the NHase activator solubility and the abolition of function is not due to a solubility change. All the mutations reduced the amounts of both NHase α and β subunits in cell extracts to the same level as in control *E. coli* which expressed NHase alone. In addition, a proportion of

Table 1
The putative binding motif CXCC is conserved in the amino acid sequences of NHase activator homologues

| Definition in database | Species | Region containing putative binding motif | Accession No. | % identity[a] |
|---|---|---|---|---|
| Nitrile hydratase activator | *Rhodococcus sp. N-771* | 66LVEMTNGCICCTLR-DLLSEISALAR90 | gi: 4126499 | |
| Nitrile hydratase activator | *Xanthomonas campestris pv. campestris str. ATCC 33913* | 71LVEFSNGCICCTLRDDLLQEVKRLAS96 | gi: 21229735 | 40 |
| P44k protein | *Rhodococcus sp. AJ270* | 65LVEMTNGCICCTLR-DLLSEISALAR89 | gi: 10129794 | 72 |
| P47K | *Pseudomonas chlororaphis B23* | 69LIEMSNGCICCTLRADLLEQISDLAR94 | gi: 483198 | 44 |
| P47K protein | *Synechocystis sp. PCC 6803* | 74MVELSNGCVCCTINEDLVEAVYKVLE99 | gi: 16332336 | 33 |
| CobW/P47K family protein | *Pseudomonas putida KT2440* | 64LVEMSNGCICCTLREDLLEEVARLAE89 | gi: 26992037 | 50 |
| Cobalamin synthesis protein/P47K | *Bacillus anthracis A2012* | 62LVEIQNGCICCTLREDLIIEVNRLVE87 | gi: 21399647 | 39 |
| Low zinc transport membrane protein | *Brucella melitensis* | 65LVEMTNGCICCTLRDDLLKEVSQLAA90 | gi: 17988523 | 46 |

[a]Percent identity to the whole sequence of NHase activator from *Rhodococcus* sp. N-771.

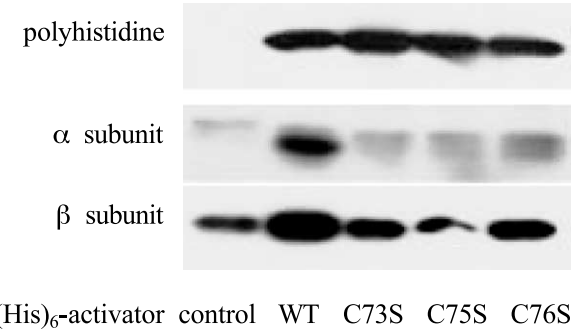


Fig. 2. Immunoblotting analysis of transformant cell extracts. *E. coli* strain BL21(DE3)pLysS in which NHase and NHase activator/mutant NHase activator were co-expressed was grown at 27°C and induced with 0.1 mM IPTG and 0.2 mM ferric citrate for 12 h. Cell extract was subjected to 8–16% SDS–PAGE and then transferred to nitrocellulose. The blots were detected with the anti-polyhistidine His probe (H-15), antiserum raised against NHase α or β subunit. In control, only NHase was expressed in *E. coli* BL21(DE3)pLysS.

NHase α and β subunits were detected to be cleaved by proteases in all samples (data not shown).

### 3.4. NHase reconstitution from the precipitate of cell lysate

A proportion of NHase α and β subunits were expressed as inclusion body from which a small amount of NHase could be reconstituted in the presence of iron in vitro. The reconstituted NHase activity from the inclusion body of *E. coli* expressing wild type NHase activator and the mutant NHase activators C73S, C75S, and C76S were 70 ± 3.2, 75 ± 4.5, 83 ± 4.2, and 67 ± 5.2 U/mg proteins, respectively. In control, the maximum NHase activity was 80 ± 4.2 U/mg proteins from the inclusion body of transformant expressing NHase alone. In the absence of iron, no active NHase was detected. As noted previously, a proportion of NHase α and β subunits were soluble in cytoplasm in the transformants expressing mutant NHase activator and the transformant expressing NHase alone. Thus, we can verify that NHase α and β subunits are translated, but the NHase activator is required to help them to get the correct conformation in the post-translational maturation process.

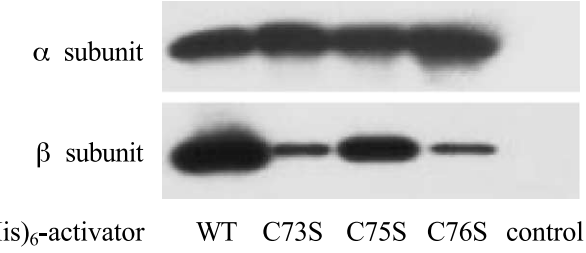


Fig. 3. Interactions between NHase and NHase activator/mutant NHase activators. *E. coli* strain BL21(DE3)pLysS in which NHase and NHase activator/mutant NHase activator were co-expressed was grown at 27°C and induced with 0.1 mM IPTG and 0.2 mM ferric citrate for 2 h. The cell extract was subjected to a Ni-NTA agarose affinity column. After washing, the proteins bound with $Ni^{2+}$ by polyhistidine residues were eluted. Aliquots of eluted solution were analyzed by immunoblotting with antiserum against NHase α or β subunit. In a control experiment, NHase activator was expressed in transformant alone, followed by affinity purification and immunoblotting.

### 3.5. Interaction between NHase and NHase activator

After NHase was co-expressed with NHase activator/mutated NHase activators in BL21(DE3)pLysS, we performed Ni-NTA metal affinity purification and immunoblotting to check the interaction between the proteins. In all eluted samples, interactions between NHase and NHase activator/mutated NHase activators were observed (Fig. 3). NHase α subunit interacting with NHase activator/mutated NHase activators was at the same level for all samples. Compared with NHase β interacting with wild type NHase activator or mutant NHase activator C75S, NHase β subunit interacting with mutant NHase activators C73S or C76S was decreased dramatically. In control experiments where NHase activator was expressed alone in *E. coli*, no NHase α or β subunit was detected. The fact that the CXCC motif does not play a predominant role indicates that another part of the NHase activator is also involved in protein–protein interaction.

### 3.6. Effect of NHase activator on the cysteine oxidization of NHase

The role of NHase activator on NHase biogenesis was further investigated by the examination on the cysteine oxidization of NHase. NHase precursor without post-translational modification can be obtained by the anaerobic assembly of recombinant NHase α and β subunits in vitro. After the NHase precursor is exposed under air, the cysteines in positions 112 and 114 will be oxidized to cysteine sulfinic acid and cysteine sulfenic acid and then show NHase activity. The presence of NHase activator does not change the cysteine oxidization kinetics and efficiency dramatically (Fig. 4). The initial point of Fig. 4 shows that NHase activator does not obviously promote cysteine oxidization under anaerobic conditions. The addition of NHase activator induces a slight and non-specific increase in maximum NHase activity yield, which may be attributed to the stabilization of proteins.

## 4. Discussion

NHase is a unique metalloprotein in which metal ion trafficking, correct protein folding and assembly, and cysteine residue oxidization is required in the post-translational bio-

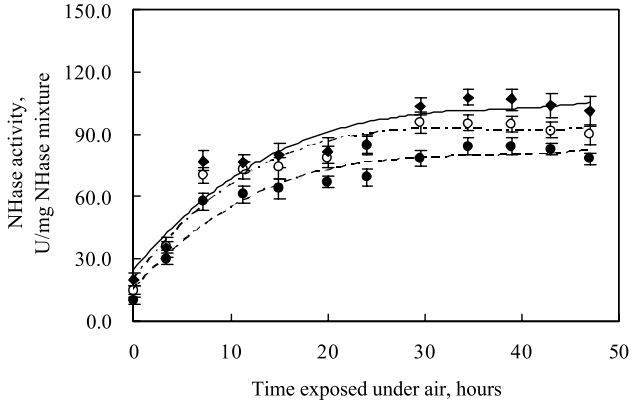


Fig. 4. Effect of NHase activator on the oxidization of NHase. NHase without post-translational modification was reconstituted by purified recombinant α, β subunits and incubated with equimolecular purified NHase activator (♦), BSA (○) or without other proteins (●) for 8 h under argon. Then the mixture was exposed under air and an aliquot of the solution was withdrawn for NHase activity assay.

synthesis. NHase activator may participate in one or several processes during the post-translational biosynthesis of NHase. Without the assistance of NHase activator, a proportion of NHase α and β subunits got soluble misfolded conformations and then would be cleaved by proteases, others were expressed as inclusion body. A small amount of unfolded recombinant NHase α and β subunits in the inclusion body can reconstitute into NHase at the diluted concentration in the presence of iron in vitro. In the absence of iron or even when recombinant NHase α and β subunits assembled alone and iron was added subsequently, no active NHase was obtained. Moreover, NO binding plays a significant role to maintain iron in the iron center of native cysteines oxidized NHase under the urea unfolding state, which is essential for the recovery of activity from urea-denatured native NHase in vitro. These observations suggest that iron acquisition and maintenance are critical for the biogenesis of NHase.

NHase activator exhibits a little enhancement for the refolding and assembly of native nitrosylated NHase in vitro, but the enhancement is non-specific and not so significant (data not shown). Thus we excluded that NHase activator plays a role as an ordinary molecular chaperone that participates broadly in de novo protein folding and recognizes hydrophobic residues and/or unstructured backbone regions in its substrate. Furthermore, NHase activator does not exhibit a critical role in the cysteine residue oxidization of NHase (Fig. 4). Accordingly, NHase activator may be an iron type metallochaperone and mainly show its vital role in assisting NHase to acquire iron in vivo.

The results of the affinity co-purification experiments provide information for the proteins' direct and indirect interactions. Interactions between NHase activator and both NHase α and β subunits were observed, although we do not know how the subunits bind to the NHase activator. The alteration of the cysteines in the CXCC motif did not affect interaction between the NHase activator and NHase α subunit. However, substitutions C73S and C76S in NHase activator significantly reduced the amount of β subunits interacting with mutant NHase activator, suggesting that in the two mutant transformants mutant NHase activator interacts with NHase α subunit directly. If NHase α subunit interacted with NHase indirectly via the NHase β subunit, only a small amount of NHase α subunit should be detected in the two mutants. Therefore, we deduce that NHase activator may interact with the NHase α subunit directly.

NHase activator was required in the iron-rich medium for the efficient expression of NHase in vivo. The reason may be attributed to competition for the free iron with other iron binding proteins or molecules and poor iron affinity of the NHase precursor. Three forms of NHase may appear in the process of NHase biogenesis, NHase precursor, cysteine oxidized NHase, and cysteine oxidized and nitrosylated NHase. The binding of iron in the active center is quite different: iron binds most tightly in cysteine oxidized and nitrosylated NHase, next most tightly in only cysteine oxidized NHase, and least tightly in NHase precursor without modification (data not shown). In point of protein stability cells may develop processes such as cysteine residue oxidization and nitrosylation as a protective mechanism for the iron binding. Accordingly, one possible explanation why NHase activator is required is to assist the poor iron binding receptor NHase precursor to get the iron in vivo. After NHase obtains iron, the iron is maintained in the active center of NHase by cysteine residue oxidization and nitrosylation. The binding of iron with cysteine oxidized NHase is an irreversible process, if the cysteine oxidized NHase loses the iron, it cannot get the iron again [32].

The sequence alignments show that all the homologues of NHase activator conserve the CXCC motif and may be involved in metal binding (Table 1), suggesting the CXCC motif is possibly a metal binding motif. Our study shows the CXCC binding motif is critical for the functional expression of NHase in *E. coli*, indicating the motif may be required in metal trafficking for NHase biosynthesis. NHase contains three conserved cysteine residues at the positions 109, 112 and 114 in the metal center. It is possible that all six cysteines in the NHase and NHase activator participate in the iron transfer process, like the copper trafficking from the copper chaperone Cu-ATX1 to target protein CCC2 in yeast, where Cu(I) transfer is carried out by the four cysteines in the copper chaperone Cu-ATX1 and target protein CCC2 [23].

In summary, co-expressions of NHase with NHase activators/mutant NHase activators in *E. coli* were investigated by activity assay and immunoblotting, followed by reconstitution and affinity co-purification experiments. We show evidence of an interaction between NHase activator and NHase structural proteins. The motif CXCC in NHase activator is critical to assist NHase to obtain the functional conformation, whereas the motif does not exhibit a predominant role in the interaction between the activator and NHase.

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