Cobalt-substituted Fe-type nitrile hydratase of *Rhodococcus* sp. N-771

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Abstract When the genes encoding α and β subunits of Fe-type nitrile hydratase (NHase) from *Rhodococcus* sp. N-771 were expressed in *Escherichia coli* in Co-supplemented medium without co-expression of the NHase activator, the NHase specifically incorporated not Fe but Co ion into the catalytic center. The produced Co-substituted enzyme exhibited rather weak NHase activity, initially. However, the activity gradually increased by the incubation with an oxidizing agent, potassium hexacyanoferrate. The oxidizing agent is likely to activate the Co-substituent by oxidizing the Co atom to a low-spin Co^{3+} state and/or modification of αCys-112 to a cysteine-sulfinic acid. It is suggested that the NHase activator not only supports the insertion of an Fe ion into the NHase protein but also activates the enzyme via the oxidation of its iron center.

Key words: Enzyme activation; Hydration; Metal substitution; Nitrile; Post-translational modification; Cysteine-sulfinic acid

1. Introduction

Nitrile hydratase (NHase; EC 4.2.1.84) catalyzes the hydration of nitriles to their corresponding amides ($RCN + H_2O \rightarrow RCONH_2$) [1–3]. NHase consists of α and β subunits, with M_r of 23 kDa each, and contains a non-heme Fe^{3+} or non-corrin Co^{3+} at the catalytic center.

Recently, the crystal structures of NHase in the active and inactive states were determined at resolutions of 2.65 and 1.7 Å, respectively [4,5]. The structure of the catalytic center is very unusual; i.e. three cysteine sulfur atoms from αCys-109, αCys-112 and αCys-114 and two amide nitrogen atoms from αSer-113 and αCys-114 are coordinated to the Fe^{3+}. The crystal structure of the inactive enzyme revealed that two cysteine ligands, αCys-112 and αCys-114, are oxidized to a cysteine-sulfinic acid (Cys-SO_2H) and a cysteine-sulfenic acid (Cys-SOH), respectively [5]. Chemically the former had been identified but the latter had been overlooked [6]. This is the first example of a metalloprotein having both cysteine-sulfenic and cysteine-sulfinic acids as ligands in a native protein.

Various spectroscopic studies revealed the metal ions of both Fe- and Co-type NHases existed as a low-spin trivalent state with similar ligand fields [7,8]. The amino acid residues around the active center in the *Rhodococcus* sp. N-771 NHase are highly conserved among both Fe- and Co-type NHases [9–14] and even in a homologous enzyme, thiocyanate hydrolase [15]. This suggests that the structure of the active center including these modifications is conserved among the enzymes belonging to the NHase family.

Irrespective of the structural conservation between Fe- and Co-type NHases, there are some differences in the biochemical characteristics between them. The Co-type NHases don't exhibit the photoreactivity, which is common among Fe-type NHases [16–19]. Fe-type NHase hydrates preferentially aliphatic small nitriles whereas the Co-type enzyme exhibits high affinity for aromatic nitriles [20–24].

Although the ion radii of Fe^{3+} and Co^{3+} are similar and structures around their binding sites are conserved between Fe- and Co-type NHases, both enzymes specifically incorporate their own metals. The Fe-type NHase incorporates only an Fe^{3+} ion in the catalytic center, and the Co-type only a Co^{3+} ion. Replacement of the bound-metal is one of the best ways to understand the functions of the metal in metalloproteins. However, there have been no reports on the metal substitutions of NHases. The crystal structure suggests that the metal center stabilizes the conformation of NHase [4,5].

It has been reported that the NHase activator, encoded in the flanking regions of Fe-type NHase genes, is indispensable for functional expression of the enzyme [9,25]. Co-type NHases from *Pseudomonas putida* 5B [26] and *Klebsiella* sp. AM1 [27] also require another accessory protein (P14K and P17K, respectively) to be functional. These proteins are supposed to act as a specific molecular chaperone for each type NHase by assisting with the insertion of the metals into the catalytic centers.

We have found that Fe-type NHase of *Rhodococcus* sp. N-771 specifically incorporates a Co ion at the catalytic center when it is expressed in *Escherichia coli* in the medium supplemented with cobalt chloride without co-expression of the NHase activator. Although the Co-substituted enzyme showed rather weak NHase activity, the activity gradually increased by the treatment with an oxidizing agent.

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Abbreviations: NHase, nitrile hydratase; $NHase_{(Fe \rightarrow Co)}$, Co-substituent of Fe-type nitrile hydratase of *Rhodococcus* sp. N-771; Cys-SO_2H, cysteine-sulfinic acid; Cys-SOH, cysteine-sulfenic acid; CM, carboxymethyl; NK24, tryptic peptide from α subunit (αAsn-105 to αLys-128); ESI, electrospray ionization; LC, liquid chromatography; MS, mass spectrometry; TPCK, *N*-tosyl-L-phenylalanyl chloromethyl ketone

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2. Materials and methods

2.1. Preparation of the recombinant NHases

The *E. coli* strains and the plasmids used in this study were described previously [9]. The plasmid, pRCN102, contains α and β subunit genes in the downstream of the T7 promoter of pET23c. The kanamycin resistant plasmid, pHSGβ was used for supplement expression of β subunit. *E. coli* JM109(DE3) cells transformed with pRCN102+pHSGβ were grown in 500 ml of Luria-Bertani (LB) medium containing ampicillin (150 μg/ml) and kanamycin (100 μg/ml) at 27°C. When the optical density at 600 nm reached 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) (0.1 mM) and the metal sources (0.4 mM ferric citrate, 0.4 mM cobalt chloride, both, or free) were added. The cells were cultured for an additional 24 h, and then harvested by centrifugation (5000×*g*, 10 min, 4°C). The crude extract was prepared as described previously [9], and the recombinant NHases were purified by almost the same procedure as that used for the native enzyme [9,20]. The purity of the recombinant NHase was estimated to be above 85% by SDS-PAGE.

2.2. Metal contents of the recombinant NHases

The preparation of samples and inductively coupled plasma mass spectrometry were performed as described previously [28].

To measure the metal content using ferrozine [29,30], all solutions were treated with Chelex-100 (Bio-Rad) (1 g/10 ml solution) to eliminate the contaminated metal ion. The proteins (20 to 40 μM, as an αβ heterodimer) were added to a final volume of 0.1 ml of 1 N HCl. To decompose the metal center, the mixture was incubated for 2 nights at 80°C. Fifty μl of 60% hydroxylamine hydrochloride was added to the mixture as a reducing agent, and the sample tube was purged with argon gas. The sample tubes were sealed and incubated at room temperature for 24 h. One hundred μl of 5 M sodium acetate and 200 μl of 1% ferrozine were added, and the absorbance of the solutions was measured with a UV-Vis spectrophotometer (UV-2100 PC, Shimadzu). In this condition, the absorption coefficients for Fe-ferrozine and Co-ferrozine were 6.045 mM^{-1} (initial concentration) cm^{-1} (562 nm) and 0.725 mM^{-1} (initial concentration) cm^{-1} (520 nm), respectively.

2.3. Oxidation of Co-substituted mutant

The protein (150 μg) was added to a final volume of 0.5 ml of 50 mM HEPES–NaOH, 44 mM *n*-butyric acid (pH 7.0) containing potassium hexacyanoferrate at the indicated concentrations (1 to 500 mM). The solutions were immediately mixed vigorously and incubated for 2 h on ice. Then, the mixtures were desalted thoroughly using a Microcon 30 (Amicon).

2.4. ESI-LC/MS

ESI-LC/MS was performed with a Finnigan LCQ ion trap mass spectrometer with an ESI probe, connected to a reversed-phase HPLC column, Mightysil C8 (i.d. 2.0×59 mm; Kanto-Kagaku) using a Hewlett Packard model 1100 liquid chromatograph. The preparation of the peptide fragment containing the metal binding site and mass spectrometry were performed as described [31].

2.5. Other methods

The concentrations of the recombinant NHases were determined with Coomassie Brilliant Blue dye reagent using bovine serum albumin as the standard [32]. Nitrile hydratase activity to methacrylonitrile was assayed according to the method described previously [9].

3. Results and discussion

3.1. Co-dependent expression of Fe-type NHase in the absence of the NHase activator

Previously we showed that NHase activator is indispensable for functional expression of NHase. When α and β subunits were expressed in *E. coli* without co-expression of the NHase activator, the subunits accumulated as inclusion bodies and the transformant showed no NHase activity. However, *E. coli* transformed with a plasmid containing the NHase activator gene in addition to the genes for α and β subunits, exhibited strong NHase activity. The amount of functional NHase in the transformant reached above 20% of the total soluble protein from the specific activity [9].

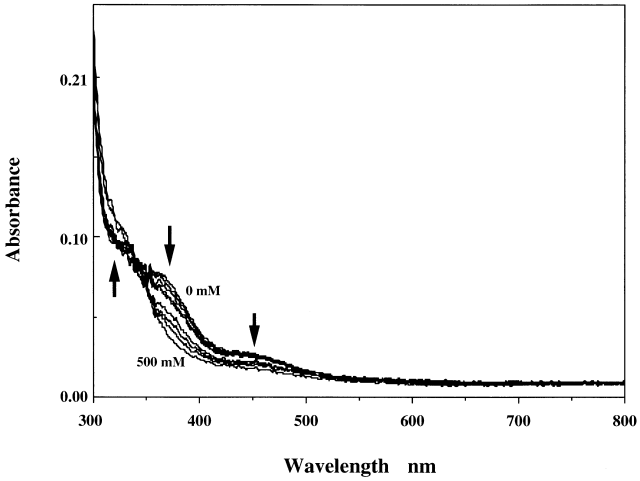

Fig. 1. Spectral change of the $NHase_{(Fe \rightarrow Co)}$ by oxidation. Each protein was dissolved at a concentration of 0.5 mg/ml in 50 mM HEPES–NaOH, pH 7.0, containing 44 mM *n*-butyric acid. The concentrations of oxidizing agent used on each spectrum were 0, 1, 2, 5, 10, 50, 100, 200 and 500 mM, respectively. All spectra were measured at room temperature with a UV-2100 PC spectrophotometer (Shimadzu).

The function of the NHase activator is unknown. It is most probable that the NHase activator helps with the folding of NHase by supporting the insertion of an Fe ion into the catalytic center. The NHase activator shows significant similarity in amino acid sequence with MagA, an ATP-dependent iron transporter, from *Magnetospillum* sp. AMB-1 [9,30]. The fact that the protein required for the expression of Co-type NHase is different from the NHase activator also supports this hypothesis [26,27].

We speculated that the functional NHase might be produced even without the NHase activator in a medium enriched with metals. We cultured the *E. coli* transformed with pRCN102 ± pHSGβ, which contains only α and β subunit genes, on LB medium supplemented with ferric citrate or cobalt chloride. Unexpectedly, the transformants showed very weak activity (2.3 units/mg protein) compared with that of transformant harboring pRCN103+pHSGβ [9] when the medium was supplemented with cobalt chloride. By contrast, no NHase activity was detected in the transformant grown on the medium enriched with Fe ion.

We suspected that the Co-substituent of Fe-type NHase had been produced in *E. coli* grown in Co-supplemented medium. The NHase was purified almost to homogeneity as judged by SDS-PAGE (data not shown). The specific activity of the purified protein was 97.0 units/mg protein when methacrylonitrile was used as the substrate, which corresponded to only 5.9% of the recombinant Fe-type NHase of *Rhodococcus* sp. N-771 [9] and 9.9% of the recombinant Co-type NHase of *Klebsiella* sp. AM1 [27].

3.2. Metal analysis of the Co-dependently expressed NHase

To confirm that the Co-dependently expressed NHase is really the Co-substituent of Fe-type NHase, the amount of the bound-metal was examined by two different methods.

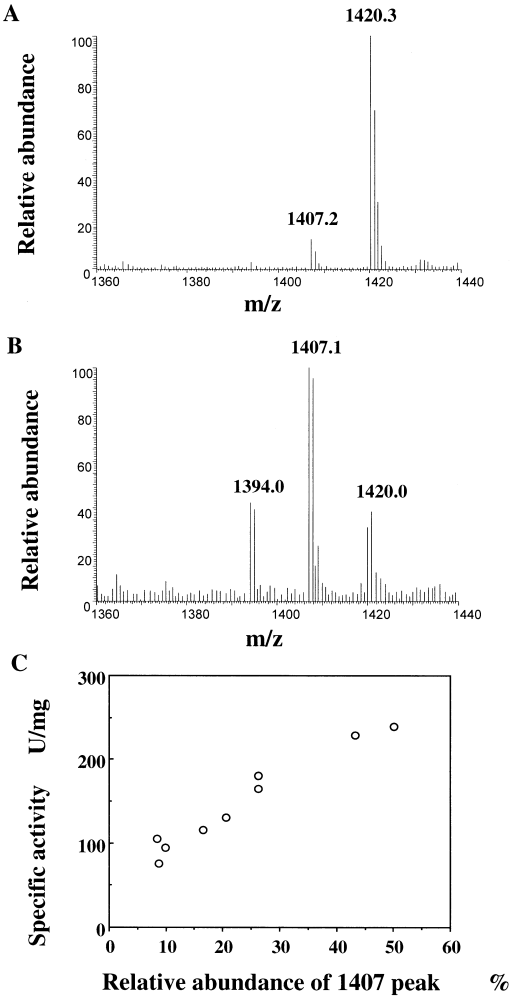

Fig. 2. Modification of αCys-112 and activation of the $NHase_{(Fe\rightarrow Co)}$. ESI-LC/MS spectra of the metal binding sites of the $NHase_{(Fe\rightarrow Co)}$ before (A) and after (B) oxidation. The mass peak with *m/z* 1407 corresponds to the $[M+2H]^{2+}$ ion of NK24 with a αCys-112-SO_2H and two CM-cysteines (calculated *m/z* value = 1406.7). The mass peak with *m/z* 1420 corresponds to that of NK24 with three CM-cysteines (calculated *m/z* value = 1419.2). The mass peak with *m/z* 1394 corresponds to that of NK24 with a CM-cysteine and two Cys-SO_2Hs (calculated *m/z* value = 1394.1). The detailed condition of ESI-LC/MS was described previously [31]. C shows the relation between the specific activity and the relative abundance of the mass peak with an *m/z* value of 1407 (R_{1407}). R_{1407} was calculated by the following equation. R_{1407} (%) = $100\times H_{1407}/(H_{1397}+H_{1407}+H_{1420})$, where H is the height of the mass peaks with *m/z* 1407, 1420, or 1394.

We first analyzed the amount of the metal in the purified NHase by inductively coupled plasma mass spectrometry, and determined the cobalt content as 0.8 mol Co atom/mol αβ heterodimer. To confirm that the enzyme does not contain Fe atoms, we measured the absorption of the metal-ferrozine complex [29]. Ferrozine formed stable complexes with Fe^{2+} or Co^{2+} ion, and showed the characteristic absorption peaks at 562 nm or 520 nm, respectively. The solution prepared from the protein hydrolysate with ferrozine exhibited only one absorption peak at 520 nm (data not shown) and the amount of Co^{2+} ion was estimated to be about 1.0 mol Co atom/mol αβ heterodimer. Thus, we concluded that the Co-dependently expressed NHase was in fact the Co-substituted Fe-type NHase (named $NHase_{(Fe\rightarrow Co)}$).

Recently, we reported that the oxidation of the cysteine residues in the active center is required for the catalytic activity of the Fe-type NHase [31]. Therefore we attempted to oxidize $NHase_{(Fe\rightarrow Co)}$ by potassium hexacyanoferrate (1–500 mM). The NHase activity of $NHase_{(Fe\rightarrow Co)}$ increased along with the concentration of oxidizing agent and reached about 240 units per mg protein. To elucidate the effect of oxidation on the $NHase_{(Fe\rightarrow Co)}$, we investigated the UV-Vis absorption spectra and post-translational modification of the $NHase_{(Fe\rightarrow Co)}$ (before and after oxidation).

3.3. *UV-Vis absorption spectra of $NHase_{(Fe\rightarrow Co)}$*

Before oxidation, the spectrum of $NHase_{(Fe\rightarrow Co)}$ showed two broad peaks at around 450 and 370 nm, being quite different from those of Fe- [33] and Co-type [14] NHases (Fig. 1). In addition, the broad peak at 710 nm, which is characteristic of the active Fe-type NHase and assigned as the $S\rightarrow Fe^{3+}$ charge transfer [34–36], was not present in the spectrum (Fig. 1). The peaks around 450 and 370 nm disappeared along with the increase of oxidizing agent concentration, and a new small absorption peak at around 320 nm appeared (Fig. 1). The spectrum of $NHase_{(Fe\rightarrow Co)}$ oxidized by 500 mM potassium hexacyanoferrate was very similar to that of the Co-type NHase from *P. putida* NRRL-18668, which contained a low-spin non-corrin Co^{3+} at the active center [14]. It has been reported that the absorption in the 300–350 nm region of the Co-type NHase from *P. putida* NRRL-18668 has an $S\rightarrow Co^{3+}$ charge transfer, because synthetic low-spin Co^{3+}-thiolate complexes show ligand-to-metal charge transfer bands at about 280 nm [37,38]. Since the Fe- and Co-type ligand environments are thought to be similar [4,7,8,14], it was suggested that the Co atom in $NHase_{(Fe\rightarrow Co)}$ was also changed to a low-spin Co^{3+} state by oxidation. In other words, it was proven that a Co ion could be incorporated into the metal center of the Fe-type NHase.

3.4. *Post-translational modification of αCys-112 in $NHase_{(Fe\rightarrow Co)}$*

We investigated the relative abundance of the post-translational modifications in $NHase_{(Fe\rightarrow Co)}$ before and after oxidation. $NHase_{(Fe\rightarrow Co)}$ was digested with TPCK-trypsin after reduction with dithiothreitol and S-carboxymethylation, and then analyzed by ESI-LC/MS. In the mass spectrum of the peptide containing the metal binding site (NK24, N^{105}VIVCSLCSCTAWPILGLPPTWYK^{128}) of $NHase_{(Fe\rightarrow Co)}$, two peaks with *m/z* 1420 and 1407 appeared (Fig. 2A). The former corresponds to the calculated *m/z* value of the $[M+2H]^{2+}$ ion of the NK24 with three carboxymethyl (CM)-cysteines (the calculated *m/z* value = 1419.2), and the latter corresponds to that of the NK24 with two CM-cysteines and a Cys-SO_2H (the calculated *m/z* value = 1406.7). From previous reports [6,9,31], it was concluded that the amino acid residue having a Cys-SO_2H modification was αCys-112. The relative abundance of the signal at *m/z* 1407 was less than 10% of that at *m/z* 1420 in the $NHase_{(Fe\rightarrow Co)}$ spectrum (Fig. 2A), while it was more than twice of that at *m/z* 1420 in the spectrum of $NHase_{(Fe\rightarrow Co)}$ oxidized by 500 mM potassium hexacyanoferrate (Fig. 2B). The relative abundance of the signal at *m/z* 1407 increased along with the increase in the specific activity (Fig. 2C). The origin of the oxygen atoms

of αCys-112-SO_2H is unknown, but it was proven that the αCys-112-SO_2H modification was induced by the oxidation of the active center (Fig. 2B). From these results, we concluded that the low-spin Co^{3+} state and the αCys-112-SO_2H stabilization in the active center were essential for the activity of $NHase_{(Fe\rightarrow Co)}$.

Additionally, it was suggested that the NHase activator not only supports the insertion of an Fe ion into NHase but also activates the enzyme via the oxidation of its iron center, because the $NHase_{(Fe\rightarrow Co)}$ was unmodified premature protein and the lack of the NHase activator gene was essential for the expression of the $NHase_{(Fe\rightarrow Co)}$.

The Fe-type NHase from *Rhodococcus* sp. N-771 has another post-translationally modified residue, αCys-114-SOH, in the active center [5]. Presently, it is impossible to directly detect the modification of αCys-114-SOH by mass spectrometry because of its lability in the preparation of the NK24 peptide. Therefore we are not sure whether the oxidized $NHase_{(Fe\rightarrow Co)}$ also has αCys-114-SOH modification. This is the subject for further investigation.

3.5. Effect of co-expressing the flanking region of Co-type NHase on expression of $NHase_{(Fe\rightarrow Co)}$

In the case of the Co-type NHase of *P. putida* 5B [26], the transformant harboring the NHase expression vector, lacking the *p14K* gene essential for expression of the functional enzyme, was able to exhibit only about 2.5% of the NHase activity of the complete gene transformant [27]. The P14K shows significant similarity in the amino acid sequence with P17K from *Klebsiella* sp. AM1 (31.9% identity), which is essential for functional expression of the Co-type NHase [27]. Therefore we also attempted to co-express P17K for the $NHase_{(Fe\rightarrow Co)}$ expression. However, co-expression of P17K had no effect on the production of functional NHase (data not shown).

3.6. Conclusion

We have succeeded in the production of active Co-substituent of Fe-type NHase of *Rhodococcus* sp. N-771 ($NHase_{(Fe\rightarrow Co)}$). Co ion was spontaneously incorporated in the enzyme without the NHase activator. But the $NHase_{(Fe\rightarrow Co)}$ exhibited rather weak NHase activity in the initial state. Oxidation of the Co atom and the αCys-112-SO_2H modification resulted in an increase in the NHase activity of $NHase_{(Fe\rightarrow Co)}$. These results indicated that the oxidation of the catalytic center was essential for the activation of $NHase_{(Fe\rightarrow Co)}$. Although it is unclear yet whether both the low-spin trivalent state of the bound-metal and the αCys-112-SO_2H stabilization were essential in the catalytic mechanism of nitrile hydration, the $NHase_{(Fe\rightarrow Co)}$ will surely be an important tool for revealing the catalytic mechanism of Fe- as well as Co-type NHases.

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