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Thiocyanate Hydrolase Is a Cobalt-Containing Metalloenzyme with a Cysteine-Sulfinic Acid Ligand

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Thiocyanate hydrolase (SCNase) catalyzes the hydration of thiocyanate to carbonyl sulfide and ammonia ($SCN^- + 2H_2O \rightarrow COS + NH_3 + OH^-$) via multiple reaction steps.[1] The enzyme was found in *Thiobacillus thioparus* THI115,[2] an obligately chemolithoautotrophic sulfur-oxidizing bacterium, which utilizes thiocyanate as a sole energy source. In nature, thiocyanate occurs ubiquitously; it is found commonly in plant tissues as thioglucosides. The organisms carrying thiocyanate-degrading ability are considered to be distributed widely.

DNA sequences of the genes coding for the α, β, and γ subunits of SCNase[3] revealed a close similarity between SCNase and nitrile hydratase (NHase) which consists of α and β subunits and catalyzes the hydration of various nitriles to the corresponding amides.[4] The NHase α subunit corresponds to γ subunit of SCNase, while the NHase β subunit corresponds to a fusion protein of β and α subunits of SCNase. NHases have unusual six-coordinated mononuclear Co(III)[5,6] or Fe(III) centers[7] as their catalytic centers. The metal center is formed in the highly conserved sequence motif, V-C1-S (Fe-type)/T (Co-type)-L-C2-S-C3 in the α subunit,[8] and three cysteine sulfurs and two amide nitrogens of the peptide backbone are coordinated to the metal.[9,10] The sixth ligand is assumed to be a solvent hydroxide or a water molecule.[11] C2 and C3 are post-translationally modified to cysteine-sulfinic acid (Cys-SO_2H) and -sulfenic acid (Cys-SOH), respectively.[10,12] NHase is the only metalloprotein that has been proved to have the oxidized cysteine ligands. Studies using the recombinant NHases showed that the post-translational modifications are essential to manifest the catalytic activity of the enzyme.[13,14] However, their detailed functions remain unknown.

The corresponding metal-binding motif is completely conserved in the γ subunit of SCNase (V^{127}-C-T-L-C-S-C^{133}).[3] Shearer et al.[15] synthesized a Co(III) complex in a mixed sulfur/nitrogen ligand environment mimicking the active center of Co-type NHase and showed that the complex binds an SCN^- and an ammonia, the substrate and product of SCNase. However, it has not been known whether SCNase contains a Co ion at its catalytic center. Here, we report that SCNase is a non-corrin Co-containing enzyme sharing characteristics very similar to those of Co-type NHases including post-translational modification of Cys^{131}. SCNase is a new member of the Co-type NHase family. Also, SCNase is the first enzyme with a Cys-SO_2H ligand other than NHases.

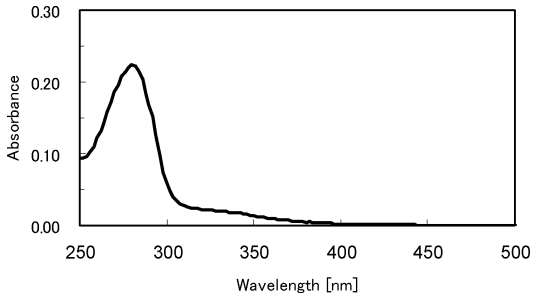

Figure 1. UV−vis absorption spectrum of SCNase from *T. thioparus* THI115. Buffer used was 50 mM Tris-HCl, pH 7.5. Protein concentration was 9.7×10^{-2} mg/mL. The spectrum was recorded by using a JASCO V-570 spectrophotometer.

We performed metal analysis of SCNase by using inductively coupled plasma (ICP) mass spectrometer (VG Elemental, VG PQΩ). ICP mass spectrum showed only cobalt peak. The amount was determined as 1.2 mol cobalt/$\alpha\beta\gamma$ heterotrimer. Since the sensitivity of ICP mass for iron is much lower than that for cobalt, the absence of iron in the enzyme was confirmed by measuring absorption of the metal−ferrozine complex. Ferrozine forms stable complex with Co^{2+} and Fe^{2+} and gives characteristic absorption peaks at 520 and 562 nm, respectively.[16] The hydrolysate of SCNase incubated with ferrozine exhibited only a broad peak at around 520 nm, similar to the complex of Co-type NHase treated with ferrozine.[14] Cobalt content of SCNase was estimated to be 0.90 cobalt/$\alpha\beta\gamma$ heterotrimer. On the basis of these results, we concluded that SCNase contains 1 cobalt ion per $\alpha\beta\gamma$ heterotrimer.

UV−vis absorption spectrum of the enzyme solution of SCNase showed an intense peak at 280 nm with a significant shoulder at around 340 nm (Figure 1). No peaks were observed in the visible region. The UV−vis absorption spectrum closely resembles that of the Co-type NHase from *Pseudomonas putida* NRRL18668[6] as well as of the Co-substituted Fe-type one of *Rhodococcus* sp. N771,[14] suggesting that the Co center of SCNase existed as a non-corrin Co^{3+} ion like Co-type NHases.[5,6] ESR characteristics of SCNase also resemble those of Co-type NHases.[5,6] Native SCNase was ESR silent. However, when SCNase was reduced with sodium

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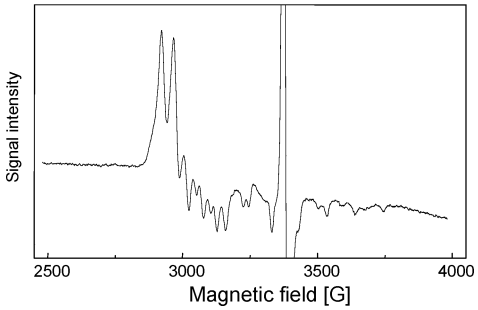

Figure 2. ESR spectrum of reduced SCNase from *T. thioparus* THI115. ESR spectra were obtained by using a Bruker E580 X-band spectrophotometer and a ER4122SHQ resonator. An Oxford-900 continuous flow cryostat with a temperature control system (Oxford, CF935) was used to keep the sample temperature at 6 K. SCNase was reduced by the addition of sodium dithionite and methyl viologen under N_2 stream. EPR conditions: microwave frequency, 9.49 GHz; microwave power, 1 mW; field modulation amplitude 16 G at 100 kHz; time constant, 81 ms; temperature, 20 K. The sharp feature at 3380 G arises from reduced methyl viologen.

dithionite and methyl viologen, it showed an ESR spectrum characteristic of low-spin Co^{2+} (Figure 2). The axial symmetric line shape gives the parameters $g_\perp = 2.254$ and 2.227 with $A_\perp =$ 82 and 80 G, respectively, and $g_{||} = 2.0027$ and 2.0063 with $A_{||} =$ 89.5 and 90.6 G, respectively. The spectra of two different Co^{2+} centers overlap with two sets of the *g*-factors. The subtle difference might arise from a different configuration in local minimum potential wells.

Finally, the post-translational modification in the SCNase metallocenter was studied by ESI−LC mass spectrometry of the tryptic digest of the SCNase that was S-carboxymethylated after reduction. The mass spectrum of a tryptic peptide containing the conserved metal-binding motif (H^{124}-V-V-V-C-T-L-C-S-C-Y-P-R-P-I-L-G-Q-S-P-E-W-Y-R^{147}) exhibited mass peaks of *m*/*z* 1478.1 and 986.0, corresponding to the $[M + 2H]^{2+}$ and $[M + 3H]^{3+}$ ions of the peptide with two S-carboxymethylated cysteine residues and 32-mass-unit increase. The calculated values of those ions are 1478.9 and 986.2, respectively. Considering the post-translational modifications of NHase,[10,12] it is strongly suggested that two of the three cysteine residues in the fragment are normally S-carboxymethylated and that the other one is post-translationally modified to Cys-SO_2H. To examine the post-translational modification in detail, MS/MS spectrum of the peak with *m*/*z* 1478.1 was examined. The assignments of the b and y series fragments clearly indicated that Cys^{131} of SCNase was post-translationally modified to increase the molecular weight by 32 Da (Figure 3). Thus, we concluded that there was a single cysteine-sulfinic acid at Cys^{131} in the catalytic center of SCNase. We could not identify whether Cys^{133} was modified to Cys-SOH by mass spectrometry because of its chemical instability. However, the modification of Cys-SOH at Cys^{133} is also plausible because of the amino acid sequence identities between SCNase and NHase at around the active center and similarity in the reaction mechanism. X-ray crystallographic studies of SCNase are in progress in our laboratory to confirm the modification of Cys-SOH at Cys^{133}.

In conclusion, we have revealed that SCNase has a low-spin Co^{3+} center for the first time. Also, we found that SCNase was the first example containing oxidized cysteine ligands other than NHases. SCNase exhibits no NHase activity, while NHase has no SCNase activity (data not shown). The differences of the catalytic mechanism as well as the substrate specificities between SCNase and NHase are subject for further investigation. However, the present study clearly indicates that SCNase is closely related to NHase and

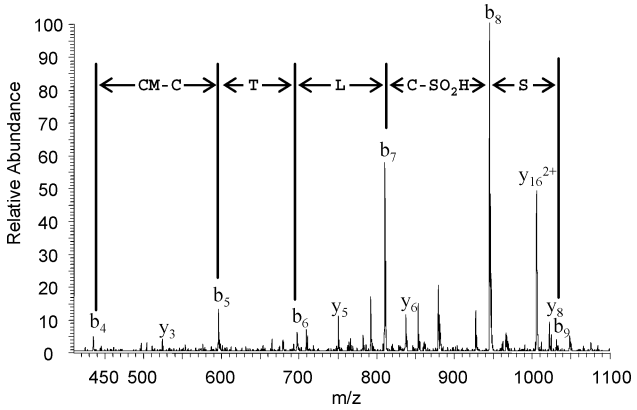

Figure 3. MS/MS spectrum of the tryptic peptide containing the metal-binding motif of SCNase (*m*/*z* 1478.1). Detailed experimental conditions are in the Supporting Information.

that SCNases and NHases form a novel non-corrin and/or non-heme protein family having post-translationally modified cysteine ligands.

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Supporting Information Available: Absorption spectrum of SCNase after treatment with ferrozine, mass spectrum of tryptic peptides of SCNase after carboxymethylation and reduction, and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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