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Two arginine residues in the substrate pocket predominantly control the substrate selectivity of thiocyanate hydrolase

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Thiocyanate hydrolase (SCNase) of *Thiobacillus thioparus* THI115 is a cobalt (Co)-containing enzyme that catalyzes the hydrolysis of thiocyanate (SCN^-), a major component of wastewater from coke oven factories, to carbonyl sulfide and ammonia. Although SCNase exhibits high structural similarities to Co-type nitrile hydratase (NHase), including a unique Co^{3+} catalytic center with two oxidized Cys ligands, both SCNase and NHase exclusively catalyze only their own substrates. Based on the differences in the substrate-binding pockets of these enzymes, βArg90 and γArg136 of SCNase, with side chains extending toward the pocket, were separately substituted with Phe and Trp, the corresponding residues, respectively, in Co-type NHase. Both SCNase βArg90 and SCNase γArg136 mutants showed no SCN^- hydrolysis activity but did catalyze the hydration of nitriles. The estimated k_{cat} values ($\sim 2\ s^{-1}$) corresponded to approximately 0.2% of that of Co-type NHase for nitrile hydration and approximately 3% of that of wild-type SCNase for SCN^- hydrolysis. The crystal structure of SCNase γR136W is essentially identical to that of the wild-type, including the Co^{3+} center having Cys oxidations; the size of the substrate pocket was enlarged because of conformational changes on the side chains of the mutated residue. Discussion of the difference in the environments around the substrate-binding pockets among the wild-type and mutant SCNases and Co-type NHase strongly suggests that βArg90 and γArg136, positioned at the top of the Co^{3+} center, predominantly control the substrate selectivity of SCNase.

[**Key words:** Thiocyanate hydrolase; Nitrile hydratase; Non-corrin cobalt; Substrate selectivity; Hydrolysis]

Thiocyanate (SCN^-) is produced commonly from thioglycosides in plants. It is also known as a major constituent of wastewater from coke oven factories and industrially degraded microbially using activated sludge processes. Thiocyanate hydrolase (SCNase) was isolated from a chemolithoautotrophic bacterium, *Thiobacillus thioparus* THI115, which degrades SCN^- to a sulfate ion to produce energy (1). SCNase catalyzes the hydrolysis of SCN^- to carbonyl sulfide and ammonia ($SCN^- + 2H_2O \rightarrow COS + NH_3 + OH^-$) and is composed of α, β and γ subunits with a non-corrin Co^{3+} catalytic center (1–3). SCNase shares high homology with the Co^{3+}- or Fe^{3+}-containing enzyme nitrile hydratase (NHase), both in amino acid sequences and structure (2–4). The SCNase γ subunit corresponds to the NHase α subunit, whereas the α and β subunits comprise the C- and N-terminal regions, respectively, of the NHase β subunit. Four SCNase αβγ hetero-trimers interact at the N-terminal extension of the β subunits to form an $(\alpha\beta\gamma)_4$ hetero-dodecamer, whereas NHase typically exists as an $\alpha_2\beta_2$ hetero-dimer (4), and the structure of the SCNase αβγ trimer is very similar to that of the NHase αβ dimer. The metal ion is bound to a short consensus motif, Cys1-Xxx-Leu-Cys2-Ser-Cys3, of the SCNase γ subunit (corresponding to γCys128 to γCys133 in SCNase) or the NHase α subunit, with similar low-spin octahedral ligand fields (3–7). In both enzymes, five proteinous ligands are common: two main chain amide nitrogen atoms from Ser and Cys3 and three sulfur atoms of Cys1–Cys3. The most striking feature of the metallocenters of both enzymes is that Cys2 and Cys3 are known to be post-translationally oxidized to cysteine–sulfinic acid (Cys–SO_2H) and –sulfenic acid (Cys–SOH), respectively (4,6,7). Cys oxygenations have been identified in various redox-related proteins, including NADH oxidase/peroxidases (8,9), peroxiredoxins (10,11) and hydrogenases (12), and are considered to play essential roles in their biological functions. However, SCNase and NHase are unique because they contain both Cys–SOH and $-SO_2H$ oxygenation at the single metal site and because they do not participate in oxidation–reduction reactions but in hydrolytic reactions. The sixth ligand site is occupied by a solvent molecule in NHase (6,7), although it appears to be vacant in SCNase (4). An anion ion from the precipitant salt is situated above the active site pocket of SCNase and interacts with the Co^{3+} ion.

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NHase catalyzes the hydration of nitriles to their corresponding amides (13) and is known as the most industrially successful enzyme because of the mass production of acrylamide and nicotinamide (14,15). Although the reaction mechanism has been extensively studied by kinetic analyses (16–18), recombinant enzymes (18–21), model complexes (22–24) and theoretical calculations (24,25), debates on the reaction mechanism continue. We found that the Fe-type NHase from *Rhodococcus erythropolis* N771 had another activity to hydrolyze isonitriles to the corresponding amines and carbon monoxide (26,27) and studied the reaction using time-resolved X-ray crystallography (27). Based on our results, we proposed a reaction mechanism in which the substrate coordinated to the metal was attacked by a water molecule activated by Cys3–SOH. Consistently, the Cys–SOH modification was found to be essential for SCNase because SCNase was inactivated when Cys3 was a normal Cys or oxidized to Cys–SO_2H (28).

These structural and functional similarities suggest that the catalytic functions of the metallocenters are conserved among SCNase and Co- and Fe-type NHases. However, there are striking differences between the catalytic features of NHase and SCNase. NHase catalyzes only hydration (14,15), whereas SCNase catalyzes hydration and subsequent hydrolysis (1). NHase catalyzes a variety of aliphatic and aromatic nitriles to their corresponding amides, whereas SCNase is known to use only SCN^- as a substrate. Moreover, NHase exhibits no SCN^- hydrolysis activity (SCNase activity), and SCNase demonstrates no nitrile hydration activity (NHase activity) (3). Previously, we noted differences in the environments around the substrate-binding pockets of these enzymes (4). The size of the pocket of the Co- and Fe-type NHase is generally small but has sufficient space for the binding of small nitrile substrates to the metallocenter. There is a hydrophobic cluster at the top of the metallocenter, which maintains the hydrophobic environment of the pocket. In contrast, the substrate-binding pocket of SCNase is much smaller and surrounded by seven Arg residues. In particular, the side chains of βArg90 and γArg136 extend toward the interior of the pocket, making the pocket highly positive, and these amino acids are replaced by hydrophobic residues in all known NHases. Therefore, we replaced βArg90 and γArg136 of SCNase with Phe and Trp, the corresponding residues in a Co-type NHase of *Pseudonocardia thermophila* JCM3060 (PtNHase) (7,20). The resulting SCNase mutants completely lost SCNase activity but exhibited NHase activity, showing that these Arg residues predominantly control the substrate and reaction selectivity of SCNase. On the basis of the results obtained, we discuss the differences in substrate selectivity between SCNase and NHase.

MATERIALS AND METHODS

Generation of the mutant SCNase and NHase The expression plasmid pSCNabgE30 containing the SCNase genes (29) was used for site-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Stratagene, CA, USA) to produce pSCNabgE30γR136W and pSCNabgE30βR90F for the expression of SCNase γR136W and SCNase βR90F mutants (SCNase γR136W and SCNase βR90F). The mutations were verified by DNA sequencing using an ABI 3130 genetic analyzer (Applied Biosystems, CA, USA). SCNase γR136W and SCNase βR90F mutants were expressed in an *Escherichia coli* strain, BL21-AI(DE3), harboring pSCNabgE30γR136W + pSAE23b or pSCNabgE30βR90F + pSAE23b. pSAE23b is the expression vector for P15K the activator protein of SCNase (29). The wild-type and mutant SCNases were purified by three successive column chromatography steps, as described previously (29) and were stored in 25 mM HEPES–NaOH (pH 7.5) at 4°C at a concentration of 10 mg/mL.

Measurement of circular dichroism spectra Circular dichroism (CD) spectra were measured at 25°C with a J-750 CD spectrophotometer (JASCO, Tokyo, Japan) equipped with a computerized data processor using a quartz cuvette with a 1.0-mm light path. Each CD spectrum was measured after 5 min preincubation, and four scans from 250 to 190 nm were averaged. The data were processed by smoothing with the simple moving average method using the software attached in the operation system. The buffer used was 20 mM sodium phosphate buffer (pH 7.5), and the concentration of each enzyme was 0.10 mg/mL.

SCN^- hydrolysis activity measurements The SCN^- hydrolysis activity was determined by measuring the absorbance at 420 nm derived from reaction of ammonia produced and Nessler's reagent (Wako, Osaka, Japan) using a Cary 50 spectrophotometer (Varian, CA, USA). The reaction was performed for 10 min at 30°C, and 40 mM potassium thiocyanate (Wako) was used as the substrate. One unit of activity was defined as the quantity of SCNase that produces 1.0 μmol of ammonia per minute (29).

Nitrile hydration activity measurement For the detection of nitrile hydration activity, 2.3×10^{-1} μg of the wild-type or mutant SCNase was added to 500 μL of the following assay mixtures: 100 mM potassium phosphate (pH 7.5) containing methacrylonitrile at a concentration of 40 mM and incubated for 1, 5, 10, 30 and 60 min at 27°C or containing crotononitrile at a concentration of 40 mM and incubated for 10 min at 27°C. The reaction was terminated by the addition of 500 μL of 3 M HCl, and the protein removed by centrifugation at 20,400 ×g for 10 min. A 100-μL aliquot of the supernatant fraction was analyzed for its absorbance at 224 nm by reversed-phase high-performance liquid chromatography (HPLC) at a flow rate of 1.0 mL/min using an ODS-80TS column (4.6 mm × 150 mm; Tosoh, Tokyo, Japan) connected to a Gulliver 1500 Intelligent HPLC system (JASCO). Solvent A was 5 mM H_3PO_4 (pH 2.9), and solvent B was CH_3CN (Sigma–Aldrich, MO, USA). The column was equilibrated with 99% solvent A and 1% solvent B, and the samples were eluted isocratically with the same buffer. For the determination of the kinetic parameters, methacrylonitrile was used as the substrate: 4.0 μg of βR90F or 6.0 μg of γR136W was added to 1.0 mL of the assay mixture, 100 mM potassium phosphate (pH 7.5) containing methacrylonitrile at concentrations of 1, 10, 20, 30, 40, 50, 75, 100 or 150 mM at room temperature. The reaction rate was obtained by continuously monitoring the absorption of the product, methacrylamide, at 224 nm with $\varepsilon_{224} = 2.52\ mM^{-1}\ cm^{-1}$ using a JASCO V-570 spectrophotometer (JASCO). Each activity measurement was obtained from the velocity from 1500 to 2000 s after the initiation of the reaction. The kinetic parameters were determined from an Eadie–Hofstee plot. One unit of activity was determined as the quantity of NHase that produces 1 μmol of methacrylamide per minute.

Crystallization of SCNase γR136W Crystals of SCNase γR136W were grown using the vapor-diffusion hanging-drop method at 4°C. A 2-μL sample of the mutant SCNase at a concentration of 20 mg/mL protein in 25 mM HEPES–NaOH (pH 7.5) was mixed with an equal volume of the precipitant solution [0.10 M potassium phosphate (pH 7.2) and 1.4 M sodium potassium tartrate] and equilibrated against 0.60 mL of the same precipitant solution. Crystals with dimensions of approximately $0.2 \times 0.2 \times 0.2\ mm^3$ grew within 3 weeks at 4°C.

X-ray data collection, structure determination and refinements Before the X-ray diffraction data collection, the crystals were soaked in the corresponding precipitant solutions containing 640 g/L of sucrose as a cryo-protectant and flash-frozen at 100 K by exposure to an N_2 stream. The diffraction data were collected using a Quantum 315 CCD detector (ADSC) at beamline NE-3 ($\lambda = 1.000$ Å) of the Photon Factory (Tsukuba, Japan) at 95 K. The data set was indexed, merged and scaled using the HKL2000 program suite (30). Molecular replacement was performed with MOLREP (31) in the CCP4 program suite (32) using the structure of native SCNase [Protein Data Bank (PDB) ID 2DXC] (4) as the initial coordinates. The models obtained were improved by iterative cycles of crystallographic refinement using REFMAC5 (33) in the CCP4 program suite and by manual model rebuilding using Coot (34). The models were cross-validated by the SigmaA-weighted electron density maps (35) calculated with both $2\ mF_{obs} - DF_{calc}$ and $mF_{obs} - DF_{calc}$ coefficients. The refinements were performed using a maximum likelihood target with bulk solvent corrections. During the structure refinement, approximately 5% of the amplitude data were set aside to monitor the progress of the refinement using the R_{free} factor. Solvent water molecules were gradually introduced if the peaks that were contoured at more than 4.0 σ in the $mF_{obs} - DF_{calc}$ electron density were in the range of a hydrogen bond. The data collection and refinement statistics are listed in Table 1. The figures in this paper were prepared with the programs PyMOL (http://pymol.sourceforge.net/). The structural data have been deposited in the Protein Data Bank under the accession number 3VYG.

RESULTS

Preparation and characterization of the mutant SCNases Both SCNase βR90F and γR136W were purified in the same way as the wild-type enzyme. We attempted to express the SCNase βR90F/γR136W double mutant in *E. coli*, to no avail. Each mutant enzyme eluted at the same retention time as the wild-type in size-exclusion chromatography. The CD spectra of the mutant enzymes are very similar to those of the wild-type (Fig. S1), thus the mutations did not affect the overall structure of the enzyme. Fig. 1 shows the UV–Vis absorption spectra of the apo- and holo-forms of wild-type SCNase and of the mutant enzymes. The UV–Vis absorption spectrum of SCNase in holo-form is very similar to