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Structural Basis for Catalytic Activation of Thiocyanate Hydrolase Involving Metal-Ligated Cysteine Modification

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Abstract: Thiocyanate hydrolase (SCNase) is a member of a family of nitrile hydratase proteins, each of which contains a unique noncorrin cobalt center with two post-translationally modified cysteine ligands, cysteine-sulfenic acid or -sulfenate (Cys-SO(H)), and cysteine-sulfininate (Cys-SO_2^-), respectively. We have found that a partially matured recombinant SCNase was activated during storage. The crystal structures of SCNase before and after storage demonstrated that Cys-SO_2^- modification of γCys131 proceeded to completion prior to storage, while Cys-SO(H) modification of γCys133 occurred during storage. SCNase activity was suppressed when γCys133 was further oxidized to Cys-SO_2^-. The correlation between the catalytic activity and the extent of the γCys133 modification indicates that the cysteine sulfenic acid modification of γCys133 is of primary importance in determining the activity of SCNase.

Introduction

Thiocyanate hydrolase (SCNase; EC 3.5.5.8) is a bacterial 240 kDa enzyme that catalyzes the degradation of thiocyanate to produce carbonyl sulfide and ammonia ($SCN^- + 2H_2O \rightarrow COS + NH_3 + OH^-$).[1] SCNase is composed of four α, β and γ subunits $(\alpha\beta\gamma)_4$; their amino acid sequences are highly conserved with those of nitrile hydratase (NHase).[2] NHases are enzymes that possess unique and complex reaction centers, and they are used in industrial mass production of acrylamide and nicotinamide.[3,4] The active centers of NHases contain a redox-inactive, mononuclear noncorrin Co(III) or nonheme Fe(III) center consisting of the short metal binding motif C1-X-L-C2-S-C3. In all known NHases, the ligand sphere is completely conserved. Two main-chain amide nitrogen atoms of S and C3 residues and three sulfur atoms of C1, C2 and C3 are coordinated in a distorted octahedral geometry together with an exogenous molecule. In addition, C2 and C3 are post-translationally modified to cysteine-sulfinic acid (Cys-SO_2H) and cysteine-sulfenic acid (Cys-SOH), respectively.[3,5] Cys-SO_2H and/or Cys-SOH modifications are found in a variety of proteins such as peroxiredoxins,[6,7] hydrogenases[8,9] and NADH peroxidases[10,11] and are likely to play diverse roles in biological systems. However, NHase family enzymes are the only proteins that possess both Cys-SOH and Cys-SO_2H modifications as metal ligands. C2 was shown to exist as Cys-SO_2^- both by FTIR[12] and by K-edge X-ray absorption spectroscopy (XAS),[13] in combination with density functional theory (DFT) calcula-

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tions. In contrast, the protonation state of C3 remains controvertible (Cys-SO(H)) because C3 was assigned as Cys-SO^- by FTIR but was demonstrated to be deprotonated by XAS. The NHase iron center exhibits high affinity for nitric oxide, the association and dissociation of which is photoregulated.[14−16] SCNase also has a low-spin Co(III) bound to C128-T-L-C-S-C133 of the γ subunit.[17]

We recently determined the crystal structures of native and apo-SCNases.[18] The structural framework of native SCNase $\alpha\beta\gamma$ heterotrimer closely resembles that of NHases including the metallocenter and the post-translational modifications of the cysteine ligands, γCys131-SO_2^- and γCys133-SO(H). However, the extrinsic solvent ligand proximal to the cobalt ion seems to be less tightly associated in contrast to those of NHases. Apo-SCNase is structurally identical to the native enzyme except for the absence of the metal and cysteine modifications. Considering the close structural similarity of these two enzymes, the metallocenters of each may contribute to catalysis in a similar way.

From the crystal structures of Fe- and Co-type NHases, several plausible catalytic mechanisms were proposed.[19−21] The most widely accepted model is that nitriles coordinate to the metal to facilitate the nucleophilic attack of a water molecule on the nitrile carbon. In the other mechanisms, a water molecule activated by the metal directly or indirectly attacks nitriles trapped in the substrate binding pocket. In all cases, the metal is suspected to function as a Lewis acid. Holz and his colleagues have studied the pH and temperature dependence of the kinetic parameters of Co-type[22] and Fe-type[23] NHases and, on the basis of the nitrile-coordination model, have proposed that the serine ligand (corresponding to γSer132 in SCNase) and a strictly conserved tyrosine residue (corresponding to βTyr108 in SCNase) activated a water molecule which made a nucleophilic attack to the nitrile carbon. We have studied the functions of the oxidized cysteine residues. The Fe-containing NHase reconstituted from unmodified subunits exhibited enzymatic activity only after aerobic oxidation of its cysteine ligands.[24] A nitrile compound, 2-cyano-2-propyl hydroperoxide, inactivated the Fe-NHase, likely due to specific oxidation of the Cys-SO(H) ligand to Cys-SO_2H.[25] Involvement of the oxidized cysteine ligands in the catalysis had also been suggested by some model complexes mimicking the modification patterns of the metal center of Co-NHase exhibiting nitrile hydration activity (for review, see refs 21 and 26). For example, the nitrile hydration activity of a N_3S_2-type model complex mimicking Co-type NHase, $[Co(PyPS)(H_2O)]^-$, was enhanced by oxygenation of one sulfur ligand to sulfinate.[27,28] The disulfenato compound $[Co(L\text{-}N_2SOSO)(t\text{BuNC})_2]^-$ catalyzed the hydration of acetonitrile, while the disulfinato derivative $[Co(L\text{-}N_2SO_2SO_2)(t\text{BuNC})_2]^-$ did not.[29] Recently, we found that Fe-type NHase from *Rhodococcus erythropolis* sp. N771 catalyzes the hydrolysis of isonitriles to the corresponding amine very slowly.[30] By monitoring the hydrolysis of *tert*-butylisonitrile with time-resolved X-ray crystallography,[31] we proposed the novel model that the substrate coordinated to the iron was attacked by a solvent water molecule activated by αCys114-SO(H) (corresponding to γCys133-SO(H) of SCNase). Hopmann et al.[32] examined theoretical calculations on the first-shell mechanism of nitrile hydration by Fe-type NHase and suggested that the Cys-SO(H) group might function as a catalytic base. These findings strongly suggest that the cysteine modification, in particular Cys-SO(H) modification of C3, is crucial for the catalytic activity. However, it was difficult to obtain detailed information on the role of each modification because of the difficulty in preparing an enzyme or model complex containing only one of the two modifications while still retaining the ideal coordination geometry.

Both Co-type[33] and Fe-type[34] NHases require their specific activator proteins for their functional expression. We have shown that SCNase requires coexpression of an activator protein, P15K, for its functional expression.[35] The detailed function of P15K remains unclear, but at minimum, it is necessary for incorporating cobalt ion to the metal-binding site. During recombinant expression of holo-SCNase, the Co content, catalytic activity and amount of Cys-SO_2^- modification in γCys131 depend greatly on the expression level of P15K.[35] When P15K was expressed prior to SCNase in *Escherichia coli*, SCNase accumulated as the holo-enzyme, while simultaneous expression of both proteins in *E. coli* resulted in decreased activity and low cobalt content in the enzyme.

Interestingly, the activity of SCNase expressed simultaneously with P15K dramatically increased after storage at −80 °C for 8 months, suggesting that maturation of the recombinant SCNase occurred during storage. We also found that further oxidation under air resulted in inactivation. Here we report the crystal structures of the recombinant SCNase in these states. The results directly show that the enzymatic activity of SCNase is closely

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related to the completion of Cys-SO(H) modification. Moreover, the results shed light on the maturation process of SCNase.

Experimental Section

Preparation of Recombinant SCNases. For production of recombinant SCNase, *E. coli* BL21 (DE3) (Novagen) harboring the plasmids pGE32 and pSAE30[35] was grown in LB medium containing ampicillin (100 μg/mL) and kanamycin (80 μg/mL) at 30 °C. When OD_{600} reached 0.5, 1 mM IPTG and 0.4 mM $CoCl_2$ were added to the medium. After further incubation for 5 h at 18 °C, cells were harvested. Purification was performed with successive chromatographic steps as described previously.[35] Purified SCNase was concentrated to 15 mg/mL and desalted with 10 mM potassium phosphate, pH 7.5, by ultrafiltration. An aliquot of the SCNase sample was used for crystallization and enzymatic assays. The remaining sample was frozen with liquid nitrogen and stored at −80 °C. The entire purification process was completed within 30 h after disruption of the cells.

Biochemical Assay. Catalytic activity of SCNase was determined by measuring the absorbance at 420 nm derived from reaction of Nessler's reagent (Wako, Osaka, Japan) and ammonia using Cary50 spectrophotometer (Varian, California, U.S.A.). Reactions were performed for 10−30 min at 30 °C, and 40 mM of potassium thiocyanate (Wako, Osaka, Japan) was used as a substrate. One unit of activity is defined as the quantity of SCNase that produces 1.0 μmol of ammonia per minute.

Crystallization, Data Collection and Structure Determination. Crystals were obtained at 20 °C by the hanging drop vapor diffusion method. Reservoir solution used was 0.10 M potassium phosphate (pH 7.2) containing 2.0 M ammonium sulfate for $SCNase_p$ or 0.10 M potassium phosphate (pH 6.9) containing 1.5 M Na/K tartrate for $SCNase_m$ and $SCNase_i$, respectively. Crystals in each forms appeared within 5-days. The data set for each state of the recombinant SCNase was collected under cryo-nitrogen stream using a Jupiter 210 CCD detector (Rigaku) at BL45XU in SPring-8 (Hyogo, Japan), Quantum 210 (ADSC) at NW12 or Quantum 4R at BL6A in the Photon Factory (Tsukuba, Japan), respectively. The data were indexed, merged and scaled with HKL2000 program suite.[36] The molecular replacement method was performed using the program Molrep[37] to determine each SCNase model. The structure of native SCNase (code 2DD5) was used as a search model. The resultant models were refined with the program suite CNS.[38] After iterative refinements, several additional cycles of occupancy refinements of Cys-SO_2H, Cys-SOH and cobalt ions were carried out using CNS. To verify the resultant occupancies, individual *B*-factor refinements were carried out alternately with the occupancy refinements. Figures in this paper were prepared with the programs PyMOL [http://pymol.sourceforge.net]. The stereochemical parameters of Cys-SO(H) and Cys-SO_2^- as well as exogenous solute molecules were taken from the output value of PRODRG.[39] The coordinates and structure factor data have been deposited in the PDB with accession codes 2DXB ($rSCNase_p$), 2DXC ($rSCNase_m$), and 2ZZD ($rSCNase_i$).

Results

The Metal-Site Structures before and after Activation. Recombinant SCNase, obtained by simultaneous expression of P15K and its α, β and γ subunits, contained 0.56 mol Co/mol $\alpha\beta\gamma$ heterotrimer. It exhibited an enzymatic activity of 8.0 units/mg at 10 days after purification, corresponding to 25% of that of the native enzyme.[35] We found that the activity was elevated up to 30 units/mg after storage at −80 °C for 8 months. We termed the recombinant SCNase 10 days after purification as $rSCNase_p$ and the spontaneously activated one as $rSCNase_m$. Subsequently, we determined the crystal structures of $rSCNase_p$ and $rSCNase_m$ (Table 1). Both structures were nearly identical to that of native SCNase, except for the structures around the metallocenter.

The $F_o - F_c$ omit electron density map of the region surrounding the Co center of $rSCNase_p$ is shown in Figure 1a. The Co ion was in a distorted square-pyramidal geometry that was very similar to that of native SCNase. The endogenous phosphate ion was stabilized by the positive nature of the substrate-binding pocket. When the cutoff was set to 4.5σ, two significant electron densities corresponding to the sulfinic group of γCys131-SO_2^- were observed. On the other hand, the electron density proximal to the sulfur atom of γCys133 was barely visible even when the threshold was lowered to 1.5σ. After iterative refinements, the occupancies of the Co atom and the Oδ1 and Oδ2 atoms of γCys131-SO_2^- converged with 0.59, 0.68 and 0.84, respectively, while that of the Oδ atom of γCys133-SO(H) was only about 0.20 (Figure 2). In contrast, in $rSCNase_m$, the electron densities corresponding to the three oxygen atoms of the $-SO_2^-$ and −SO(H) groups were clearly observed proximal to the sulfur atoms of γCys131 and γCys133, respectively (Figure 1b). The occupancy of the Co atom converged to 0.60, and those of the Oδ1 and Oδ2 atoms of γCys131-SO_2^- and the Oδ atom of γCys133-SO(H) converged to 0.69, 0.85 and 0.79, respectively (Figure 2). The occupancies of the Oδ1 and Oδ2 atoms of γCys131-SO_2H in $rSCNase_m$ were almost identical to those in $rSCNase_p$, while the occupancy of the Oδ atom of γCys133-SO(H) was about 4 times larger than that of $rSCNase_p$. The occupancy of the Co ion in $rSCNase_p$ and $rSCNase_m$ coincided well with the cobalt content; however, those of the oxygen atoms of two cysteine ligands were calculated slightly larger. Since the apo-SCNase has no cysteine oxygenation,[18,35] the occupancies of the oxygen atoms are likely to be overestimated compared to the actual modifications. This may be due to the relatively low accuracy in the determination of the occupancies of small atoms such as oxygen. However, the significant difference in the electron densities of the Oδ atom of γCys133-SO(H) between $rSCNase_p$ and $rSCNase_m$ clearly indicates that the Cys-SO(H) modification occurred in only a small fraction of the enzyme until 10 days after purification and increased during storage at −80 °C for 8 months.

Structure of Air-Inactivated SCNase. When recombinant SCNase, containing γCys131-SO_2^- and γCys133-SO(H) modifications in the cobalt center, was stored at 20 °C under aerobic conditions for 4 months, no enzymatic activity was detected. To clarify the reason for the loss of activity, the crystal structure of the inactivated SCNase (termed as $rSCNase_i$) was determined. $rSCNase_i$ maintained the identical fold (Table 1), but two notable changes occurred at the metal center of $rSCNase_i$ (Figure 1c). First, γCys133-SO(H) was further oxygenated to Cys-SO_2^-. The electron density corresponding to Oδ2 of γCys133-SO_2^- is positioned at the opposite side of the substrate binding pocket with a distance of 1.5 Å from the Sγ atom. In previous mass spectrometric measurements of recombinant holo-form of SCNase, 74% of the enzymes had only one Cys-SO_2^- modification at γCys131 (the Cys-SO(H) modification cannot be detected because of its chemical instability), but about 13% had two Cys-SO_2^- modifications at γCys131 and γCys133. Thus, it is likely that oxygenation of γCys133-SO(H) to Cys-SO_2^- occurred

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Table 1. Data Collection and Refinement Statistics

data set name	$rSCNase_p$	$rSCNase_m$	$rSCNase_i$
Crystallographic Data			
beamline	SPring-8 BL45PX	PF NW12	PF BL6A
space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
unit cell parameters (Å)	$a = 114.9$, $b = 245.1$, $c = 244.7$	$a = 114.9$, $b = 170.5$, $c = 175.2$	$a = 115.0$, $b = 170.8$, $c = 175.2$
number of molecules (au)	2	1	1
wavelength (Å)	1.00	1.00	1.00
resolution range (Å)[a]	50.00−2.25 (2.33−2.25)	50.00−1.90 (1.97−1.90)	50.00−1.78 (1.89−1.78)
number of observations	1,511,813	1,539,486	1,677,550
number of unique reflections[a]	322,968 (31,875)	263,483 (24,895)	319,150 (30,773)
completeness (%)[a]	99.7 (99.4)	97.6 (93.1)	97.3 (94.6)
R_{sym} on intensities (%)[a,c]	9.9 (20.8)	4.4 (15.8)	5.1 (29.0)
mean $I/\sigma(I)$	8.5	19.7	13.9
Refinement Statistics			
resolution range (Å)[a]	49.4−2.25 (2.34−2.2)	41.85−1.90 (2.02−1.90)	40.76−1.78 (1.89−1.78)
number of reflections (total)[a]	316,929 (28,477)	260,988 (36,549)	309,775 (43,087)
number of reflections (test)[a]	31,764 (3,218)	26,126 (4,149)	31,002 (4,864)
R_{cryst} (%)[a,b,d]	17.1 (19.5)	17.7 (18.8)	15.0 (18.7)
R_{free} (%)[a,b,d]	19.8 (23.3)	19.6 (20.8)	15.9 (19.6)
average B factor (Å^2)	20.8	16.6	19.7
Number of Molecules in the Model			
peptide atoms	31,390	15,714	15,749
water molecules	2,941	2,532	3,400
hetero atoms	48	44	92
Structure Validation			
rmsd from ideal values			
bond lengths (Å)	0.006	0.005	0.006
bond angles (deg)	1.4	1.4	1.5
Luzzati mean coordinate error (Å)	0.21	0.19	0.15
Ramachandran Plot			
most favored (%)	90.8	90.8	90.8
disallowed (%)	0.2	0.2	0.2
rmsd from native SCNase (Å)	0.337	0.241	0.263

[a] Values in parentheses are for the highest resolution shell. [b] Calculated using a test data set; 10% of total data are randomly selected from the observed reflections. [c] $R_{sym} = \Sigma|I_i - \langle I_i\rangle|/\Sigma|I_i|$ where I_i is the scaled intensity of the ith measurement, and $\langle I_i\rangle$ is the mean intensity for that reflection. [d] R_{cryst} and $R_{free} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$ where F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes, respectively.

gradually under aerobic conditions. Second, an electron density assignable to a mono-oxygen atom was found at the sixth coordination site of the cobalt ion, facing the substrate-binding pocket. We assigned the electron density to a bound water molecule. We should note that in all other SCNase structures determined previously, the sixth ligand site was not occupied by a solvent ligand but instead associated with solute anions involved in the crystallization solution such as sulfate, tartrate or phosphate ions. These anionic molecules are stabilized by electrostatic interactions with the wall of the substrate-binding pocket rather than by coordination with the cobalt ion.[18] In contrast, the average Co(III)-to-oxygen distance was 2.1 Å, shorter than the Co-γCys128Sγ distance, resulting in formation of a square-based bipyramidal coordination sphere with more nearly ideal configuration (Table 2).

Discussion

Enzyme Activity Is Primarily Influenced by the Oxygenation State of γCys133. The present study revealed that the enzymatic activity of recombinant SCNase correlates well with the oxygenation state of the γCys133 residue. As γCys133-SH is modified to Cys-SO(H), the enzymatic activity is raised to the extent of native enzyme. When γCys133 is further oxidized to Cys-SO_2^-, activity is suppressed. Since no apparent structural change is found between these three states other than their metallocenters, a specific oxygenation state, Cys-SO(H) for γ133, must greatly influence the catalytic activity of SCNase. Various studies using recombinant proteins,[24,40,41] a competitive inhibitor,[25] model complexes[13,21,26,42−45] and theoretical calculations[13,32,45,46] suggested the involvement of the Cys-SO(H) modification in the catalytic reaction of Fe- as well as Co-type NHases. In particular, recent time-resolved X-ray crystallographic study on the isonitrile hydrolysis activity of Fe-type NHase[31] gave the structural evidence that the Cys-SO(H) ligand behaved as the catalytic base. However, there are no studies on the enzyme or model compounds with only one modified sulfur ligand with ideal geometry, and thus most studies had been focused on the activation by simultaneous oxidation of two sulfur ligands or inactivation by oxidation to two sulfinato ligands. The structural and biochemical evidence presented in this study clearly indicates that metal-bound recombinant SCNase possessing only the γCys131-SO_2^- modification is activated by Cys-SO(H) oxygenation of γCys133-SH and

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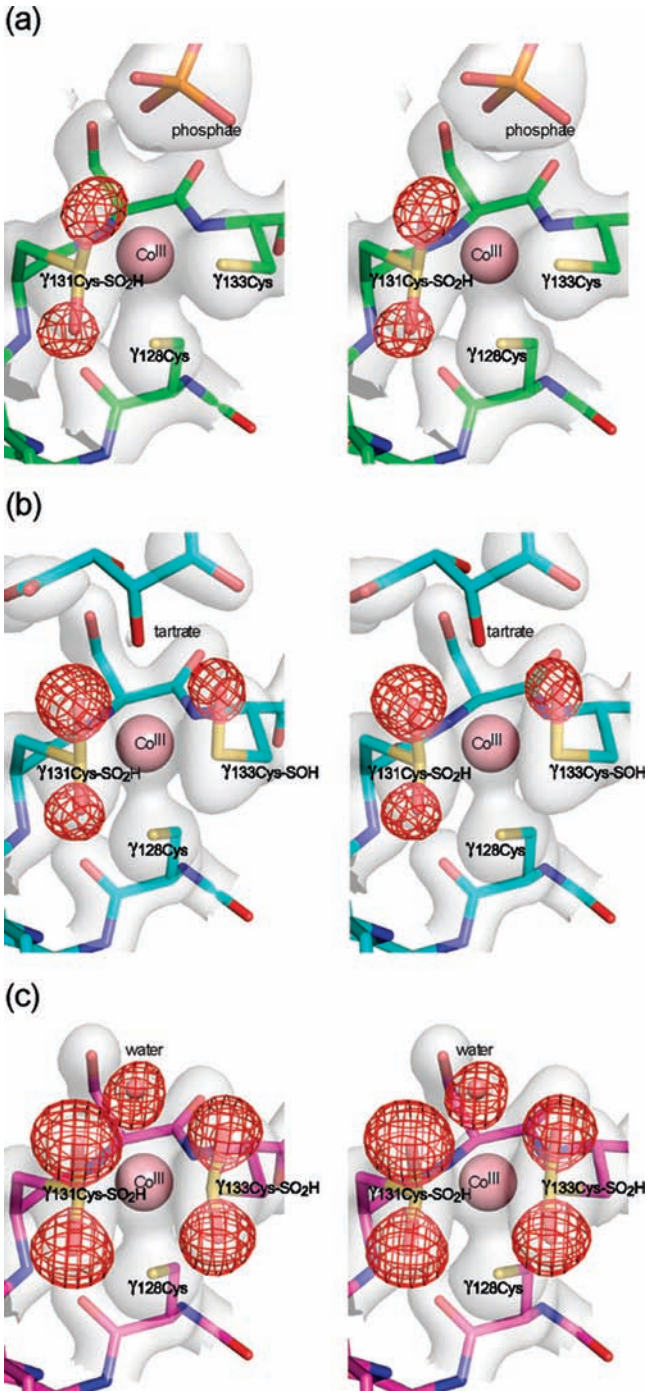


Figure 1. Comparison of the cysteine modifications in $rSCNase_p$, $rSCNase_m$ and $rSCNase_i$. Close-up views around the metallocenters of (a) $rSCNase_p$, (b) $rSCNase_m$ and (c) $rSCNase_i$ at resolutions of 2.25, 1.9 and 1.78 Å, respectively. The backbone carbons are indicated in green, cyan, or magenta, nitrogens in blue, oxygens in red, sulfurs in orange and cobalts in pink. The $2F_o - F_c$ electron density map and the nonbiased $F_o - F_c$ omit map are overlaid on the models. Oδ1, Oδ2 of γCys131-SO_2^-, Oδ atom of γCys133-SO(H) residues and the water ligand are omitted from the calculations of $F_o - F_c$ map. The $2F_o - F_c$ map is contoured with 1.5 σ in gray surfaces, and the $F_o - F_c$ map is contoured with 4.5 σ in red mesh.

inactivated by its further oxygenation to Cys-SO_2^-. Although some defective mutants retaining the same modification pattern as native enzymes are reported,[40,41] our study using intact SCNase reveals that the existence of Cys-SO(H) is the primary important determinant for activity.

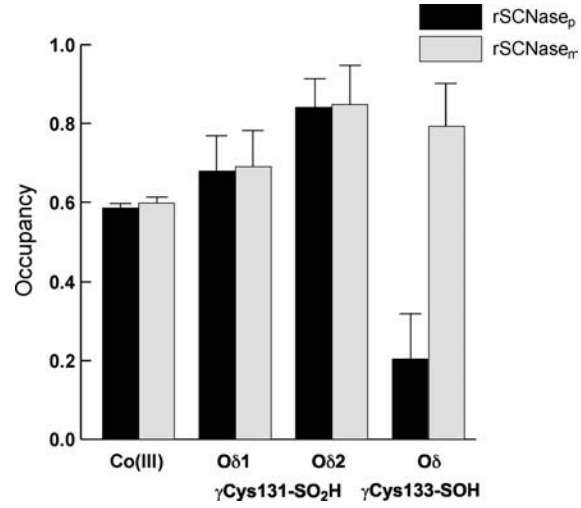


Figure 2. Refined occupancies of the cobalt(III) ion and the post-translationally modified oxygen atoms. In each column, the arithmetical averages of the equivalent sites in an asymmetric unit are shown. In $rSCNase_p$, two $(\alpha\beta\gamma)_4$ complexes are present in an asymmetric unit ($n = 8$); in $rSCNase_m$, one $(\alpha\beta\gamma)_4$ complex is present ($n = 4$). The bars indicate standard deviations. The corresponding *B*-factors of Co(III), Oδ1 of γCys131, Oδ2 of γCys131 and Oδ of γCys133 were converged to 12.8, 18.8, 16.5, and 17.6 in $rSCNase_p$, and to 9.4, 10.3, 11.5, and 17.2 in $rSCNase_m$, respectively.

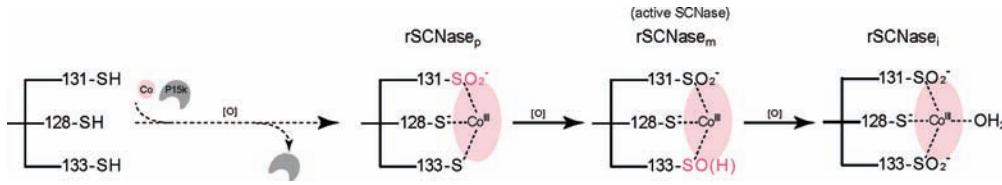
Table 2. Selected Bond Distances (Å) of the Metal Centers

	$rSCNase_p$	native SCNase	$rSCNase_i$	Co-NHase	Fe-NHase
		First Shell			
M(III)−$O_{anion/water}$	3.54	2.96	2.14	2.58	–
Fe(III)−N_{NO}	–	–	–	–	1.65
M(III)−$S\gamma_{Cys1}$	2.33	2.44	2.36	2.28	2.31
M(III)−$S\gamma_{Cys2}$	2.27	2.35	2.19	2.14	2.27
M(III)−$S\gamma_{Cys3}$	2.42	2.39	2.22	2.28	2.32
M(III)−N_{Ser}	1.88	2.18	2.12	2.09	2.10
M(III)−N_{Cys3}	1.99	1.98	1.95	1.96	2.06
$S\gamma_{Cys2}$−N_{Ser}	3.14	3.48	3.19	3.18	3.24
N_{Ser}−N_{Cys3}	2.61	2.66	2.66	2.73	2.83
N_{Cys3}−$S\gamma_{Cys3}$	2.92	3.09	2.91	2.91	2.99
$S\gamma_{Cys2}$−$S\gamma_{Cys3}$	3.39	3.35	3.19	3.16	3.32
$S\gamma_{Cys2}$−$O\delta1_{Cys2}$	1.44	1.43	1.50	1.50	1.46
$S\gamma_{Cys2}$−$O\delta2_{Cys2}$	1.50	1.50	1.43	1.42	1.46
$S\gamma_{Cys3}$−$O\delta1_{Cys3}$	–	1.49	1.43	1.54	1.53
$S\gamma_{Cys3}$−$O\delta2_{Cys3}$	–	–	1.50	–	–
		Second Shell			
M(III)−$O\delta1_{Cys2}$	3.01	3.17	3.04	2.98	3.18
M(III)−$O\delta2_{Cys2}$	3.29	3.37	3.09	3.01	3.18
M(III)−$O\delta1_{Cys3}$	–	3.14	2.96	3.00	3.16
M(III)−$O\delta2_{Cys3}$	–	–	3.30	–	–
M(III)−$O\gamma_{Ser}$	4.07	4.01	3.99	3.85	3.77
$O_{anion/water}$−$O\delta1_{Cys2}$	3.78	3.57	2.76	2.74	2.91
$O_{anion/water}$−$O\delta1_{Cys3}$	3.68	2.98	2.74	2.66	2.81
$O_{anion/water}$−$O\gamma_{Ser}$	2.73	2.88	2.78	2.89	2.96

Values are the averages between each corresponding site in the asymmetric unit, and taken from PDB entry 2DD5 for native SCNase, 1IRE for Co-NHase and 2AHJ for Fe-NHase, respectively.

Changes in Coordination Sphere of the Cobalt Center According to Oxygenation. The effects of oxygenated cysteine ligands on the electronic state of the NHase metallocenter remain one of the most important issues in the study of the catalytic mechanism of SCNase as well as NHase. Prior to the current study, there had been no protein chemical data on the relation between the Lewis acidity of the metal and its ligand oxygenations, primarily due to difficulty in preparing enzymes having only mono-oxygenated cysteine

Scheme 1. Sequential Activation Model of SCNase Focused on Cobalt Incorporation and Oxygenation[a]



[a] From ref 35 P15K is responsible for the cobalt incorporation although the detailed mechanism remains unclear.

ligand. In this study, we determined the structures of recombinant SCNases with three different cysteine oxygenation states. The fact that only rSCNase$_i$ coordinated the solvent water at the sixth site indicates that the Lewis acidity of the SCNase cobalt center increases when γCys133-SO(H) is further oxygenated to Cys-SO_2^-. Characterization of a series of N_2S_2 type model complexes ($[Co^{III}N_2S_2(t\text{BuNC})_2]^-$, $[Co^{III}N_2(SO)_2(t\text{BuNC})_2]^-$, $[Co^{III}N_2(SO_2)_2(t\text{BuNC})_2]^-$)[29,43] demonstrated that the coordination strength of *t*BuNC to the Co ion increases with an increase in the oxidation number of the sulfur ligands. Consistent with this result, characterization of Fe-type NHase analogues including sulfur K-edge XAS combined with DFT-calculations demonstrated that the oxidized sulfur ligands functioned as weaker electron donors, which would increase the Lewis acidity of the Fe(III) center.[13,45] Taken together, these data suggest that the oxidation state of the thiolato ligands is likely to determine the appropriate substitution lability of the catalytically relevant coordination site of SCNase, as well as other NHases.

Sequential Modification Process of the Metal Center. When SCNase is expressed with P15K, maturation occurs post-translationally through cobalt incorporation and specific modification of the two cysteine ligands. The structures presented in the present study suggest the likely maturation process of SCNase. On incorporation of Co ion, γCys131-SO_2^- modification proceeds to completion relatively quickly, while γCys133-SO(H) modification occurs much more slowly. Thus, the two cysteine oxygenations are likely to occur sequentially, with γCys131-SO_2^- modification occurring first and γCys133-SO(H) modification following (Scheme 1). In SCNase, the Co center is open at the bottom of the solvent-accessible channel. γCys131 faces the channel, while the Co center is located between γCys133 and the channel. It is possible that solvent accessibility of the two residues influences their oxidative sensitivities. In fact, when apo-SCNase is treated with hydrogen peroxide, γCys131 is specifically oxygenated prior to any other amino acid residues (Arakawa, T.; et al. Manuscript in preparation). Very recently, Kobayashi and his co-workers discovered that an accessory protein, NhlE, encoded downstream of Co-type nitrile hydratase genes of *Rhodococcus rhodochrous* J1 formed a complex with the α subunit (corresponding to SCNase γ subunit) together with cobalt ions, and that the cobalt-bound α subunit in the complex was exchanged with the α subunit of apo-NHase protein to form the mature NHase enzyme (termed as self-subunit swapping).[47] They have also demonstrated that NhlE assists the cobalt incorporation as well as post-translational modification of αCys112 (corresponding to γCys131-SO_2^-) to Cys-SO_2^-.[48] The view that the Cys-SO_2^- modification occurs in an early step of the Co-type NHase maturation is good agreement with the present study. P15K might function in a similar manner. However, they obtained no information on the biogenesis of the Cys-SO(H) modification because of the chemical instability of the sulfenate group. We attempted to detect sulfenic species by labeling with dimedone, a specific reagent for Cys-SOH,[49] but so far, we have failed, most likely due to the existence of a narrow and deep path to the C3 site (data not shown). In summary, our work presents the first structural evidence to date on the generation of the Cys-SO(H) modification in NHase family proteins. It strongly suggests that the cysteine modifications in SCNase occur sequentially and that Cys-SO(H) modification of γCys133 can occur in an autocatalytic manner, at least *in vitro*.

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