Structure of the Photoreactive Iron Center of the Nitrile Hydratase from *Rhodococcus* sp. N-771

**EVIDENCE OF A NOVEL POST-TRANSLATIONAL MODIFICATION IN THE CYSTEINE LIGAND***

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Nitrile hydratase (NHase) from *Rhodococcus* sp. N-771 is a photoreactive enzyme that is inactivated by nitrosylation of the non-heme iron center and activated by photodissociation of nitric oxide (NO). To obtain structural information on the iron center, we isolated peptide complexes containing the iron center by proteolysis. When the tryptic digest of the α subunit isolated from the inactive form was analyzed by reversed-phase high performance liquid chromatography, the absorbance characteristic of the nitrosylated iron center was observed in the peptide fragment, Asn105-Val-Ile-Val-Cys-(Ser-Leu-Cys-Ser-Cys-Thr-Ala-Trp-Pro-Ile-Leu-Gly-Leu-Pro-Pro-Thr-Trp-Tyr-Lys128). The peptide contained 0.79 mol of iron/molecule as well as endogenous NO. Subsequently, by digesting the peptide with thermolysin, carboxypeptidase Y, and leucine aminopeptidase, we found that the minimum peptide segment required for the nitrosylated iron center is the 11 amino acid residues from eL107 to aTrp117. Furthermore, by using mass spectrometry, protein sequence, and amino acid composition analyses, we have shown that the 112th Cys residue of the α subunit is post-translationally oxidized to a cysteine-sulfenic acid (Cys-SO2H) in the NHase. These results indicate that the NHase from *Rhodococcus* sp. N-771 has a novel non-heme iron enzyme containing a cysteine-sulfenic acid in the iron center. Possible ligand residues of the iron center are discussed.

Nitrile hydratase (NHase; EC 4.2.1.84) is a bacterial metalloenzyme catalyzing the hydration of nitriles to correspond-

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1 The abbreviations used are: NHase, nitrile hydratase; ESR, electron spin resonance; NO, nitric oxide; NK24, peptide isolated from the α subunit of the inactive NHase (αAsn105 to αLys128); 11W11, peptide isolated from NK24 (αLeu111 to αTrp117); NK24cys, NK24 isolated from the active form of the NHase; LP8, thermolytic peptide of NK24cys (αLeu111 to αPro117); FAB-MS, fast atom bombardment-mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; FLEC, (+)-1-(9-fluorenyl)ethyl chloroformate; MGD, N-methyl-n-glucamine dithiocarbamate; TPCK, L-1-tosylamide-2-phenyl ethyl chloromethyl ketone.

2 J. Honda, T. Nagamune, and I. Endo, unpublished results. This bacterium was previously known as *Brevibacterium* sp. R312 (40).

3 M. Yohda and I. Endo, unpublished results.
colleagues\textsuperscript{4} digested the inactive NHase with Pronase, and obtained a peptide containing chromophore with an absorption peak characteristic of the inactive form. These findings suggest that the iron atom binds with a short specific region of the $\alpha$ subunit. In the present study, we analyzed the iron-binding site through proteolytic cleavage of the $\alpha$ subunit followed by reversed-phase HPLC, and found that the nitrosylated iron center complex consists of a segment of 11 amino acids of the $\alpha$ subunit, which contains the highly conserved Cys cluster. Furthermore, we have shown by mass spectrometry, protein sequence, and amino acid composition analyses that a Cys residue in this region is post-translationally oxidized to a cysteine-sulfenic acid (Cys-SO$_2$H). Based on the results, we will discuss the structure of the photoreactive non-heme iron center of the NHase in the inactive form.

EXPERIMENTAL PROCEDURES

Preparation of the Inactive and Active Forms of the NHase and the $\alpha$ Subunit—Dark-inactivated NHase was prepared as described\textsuperscript{21}. For photoinactivation, the NHase in the inactive form was irradiated with a photoreactor lamp (500 W SPOT, Toshiba) for 15 min in an ice bath. The $\alpha$ subunit was isolated from the inactive form by anion-exchange chromatography in the presence of 6 M urea in the dark, while the isolation from the active form was carried out under light\textsuperscript{21}. The $\alpha$ subunit isolated from the inactive form was digested with thermolysin (0.02 mg/ml) for 6 h at 37 °C. The N-terminal half of the $\alpha$ subunit gene was amplified by polymerase chain reaction to introduce an NdeI site at the initiation codon. The fragment was digested by NdeI and PstI, whose digest site is located just upstream of the complementary polymerase chain reaction-primer binding site, and subcloned into pET-3c with the remainder of the $\alpha$ subunit gene. Escherichia coli (BL21(DE3)), was transformed with the constructed expression vector, pRCMa, and the recombinant $\alpha$ subunit ($\alpha_{\text{rec}}$) was expressed as inclusion bodies. Cells were resuspended in 50 mM Tris-HCl, pH 7.5, and disrupted by sonication. After removal of the supernatant by centrifugation (40,000 $\times g$, 20 min), the precipitate was washed with 1 M sucrose and then 2% Triton X-100 and 100 mM EDTA. The washed precipitate was solubilized with 50 mM Tris-HCl, pH 7.5, containing 6 M urea and 2 mM 2-mercaptoethanol and applied to an anion-exchange Q Sepharose column (Pharmacia, 2.5 cm diameter $\times$ 10 cm) equilibrated with the same buffer. $\alpha_{\text{rec}}$ was eluted with a 0–500 mM NaCl linear gradient at a flow rate of 1.5 ml/min. $\alpha_{\text{rec}}$ was estimated to be more than 99% pure by SDS-polyacrylamide gel electrophoresis, and stored at 4 °C as a suspension in 60% saturated ammonium sulfate.

Preparation of the Peptide Fragments from the Inactive Form of the NHase—All manipulations were executed in the dark to avoid photodissociation of NO from the iron center. The $\alpha$ subunit isolated from the inactive NHase was thoroughly desalted using a Centriprep-10 (Amicon) with 20 mM Tris-HCl, pH 8.0, containing 10 mM CaCl$_2$ and 2 mM 2-mercaptoethanol. The $\alpha$ subunit at a final concentration of 2–2.5 mg/ml in 500 $\mu$l was treated with TPCK-trypsin (14 $\mu$g) for 2 h at 37 °C. The digest was subjected to reversed-phase HPLC with a Capcell Pak C18 column (4.6 $\times$ 250 mm, Shiseido). Solvent A was 20 mM Tris-HCl, pH 7.5, containing 2 mM CaCl$_2$ and 2 mM 2-mercaptoethanol, and solvent B was 20% A + 80% acetonitrile. The content of B was increased as follows: 0–2 min, 0%; 2–4 min, 0–20%; 4–24 min, 20–60%; 24–26 min, 60–100%; 26–28 min, 100%. The flow rate was 1.0 ml/min. For quantification of the iron atom in the peptide, 5 mM ammonium acetate, pH 7.5, was used as solvent A.

To minimize the size of the iron center complex peptide, the trypptic peptide was further digested. The peptide (1.35 $\mu$g) in 20 mM Tris-HCl, pH 7.5, was treated with 1 $\mu$m of thermolysin for 1 h at 37 °C, and then with carboxypeptidase Y (1 $\mu$g) and leucine aminopeptidase M (1 $\mu$g) for 12 h at 37 °C.

Preparation of the Peptide Fragments from the Active NHase—The $\alpha$ subunit isolated from the active enzyme was cleaved with trypsin without being shielded from light. The digest was separated by reversed-phase HPLC with an Aquapore RP300 column (4.6 $\times$ 100 mm, Perkin-Elmer Applied Biosystems). Solvent A was 0.09% trifluoroacetic acid, and solvent B was 0.075% trifluoroacetic acid containing 80% acetonitrile. The column was equilibrated with 100% solvent A and eluted with a linear gradient of 0 to 80% solvent B over a period of 16 min at a flow rate of 0.5 ml/min.

Mass Spectrometry Measurements—The molecular masses of proteins and peptide fragments were determined by fast atom bombardment-mass spectrometry (FAB-MS) or matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). FAB-MS spectra were obtained with a JEOL JMS-HX110 mass spectrometer operated at 10-kV accelerating voltage and a resolution of 1:3000. Ionization was carried out using xenon gas as the primary beam with the energy of 6-keV in the positive mode. Samples were mixed with 0.1% trichloroacetic acid in 3-nitrobenzyl alcohol/thioglycolic acid (1:1:1). MALDI-TOF MS was performed on a Reflex (Bruker) equipped with a delayed extraction ion source. Samples were prepared by mixing 0.5 $\mu$l of the 10 mg/ml 2,5-dihydroxybenzoic acid matrix solution with 0.5 $\mu$l of the sample dissolved in 33% acetonitrile, 0.07% trifluoroacetic acid on the target. Positive ion mass spectra were acquired in reflectron mode at 28.5-kV accelerating voltage. Mass scale was calibrated using commercially available peptidest.

Identification of the Modification of $\alpha$ Cys12—An aliquot of the irradiated NHase (500 μg) was digested with carboxypeptidases A and Y, and then treated with trypsin (5 μg). The carboxymethylated NK24 was isolated by reversed-phase HPLC as described above. After removal of acetonitrile in a vacuum centrifuge and adjustment of the pH value to 8.0, the carboxymethylated NK24 was cleaved with thermolysin (1.0 μg) at 37 °C for 6 h, and then analyzed by reversed-phase HPLC with a Superher 60RP select B column (2.1 $\times$ 125 mm, Merck). Solvent A was 0.09% trifluoroacetic acid, and solvent B was 0.075% trifluoroacetic acid containing 80% acetonitrile. The column was equilibrated with 20% solvent B and eluted with a linear gradient from 20 to 55% solvent B over a period of 17.5 min at a flow rate of 0.2 ml/min. After removal of acetonitrile in a vacuum centrifuge and adjustment of the pH to 8.0, the fraction containing an octapeptide, LP$_{8}$ (αLeu$_{1}$ to αPro$_{8}$), was incubated with leucine aminopeptidase M (0.02 μg) for 6 h at 37 °C. Once dried in a vacuum centrifuge, the digest was dissolved in 5 μl of acetonitrile and incubated with 5 μl of 18 mM (+)-1-(9-fluorenylethyl chlorofluorocate (FLEC) (23) in acetone for 10 min at room temperature. The reaction was terminated by addition of 10 μl of 100 mM trypthophan in 100 mM borate buffer at pH 9.0. The sample solution was mixed with 10 μl of 9 N acetic acid and 40 μl of 100 mM sodium acetate buffer, pH 4.0, and then subjected to reversed-phase HPLC with a PEGASIL ODS-3 column (4.6 $\times$ 250 mm, Sera) equilibrated with 0.1% sodium acetate (NaOAc) buffer, pH 4.0, NaOAc/acetonitrile/tetrahydrofuran (77/15/8, v/v/v). The column was isocratically run with the same buffer at a flow rate of 0.6 ml/min.

Other Procedures—The concentration of the NHase was estimated by the absorbance of the inactive form at 280 nm ($\varepsilon_{280}$ = 1.7 ml·mg$^{-1}$·cm$^{-1}$)\textsuperscript{21}. The amino acid composition were analyzed with a 477A protein sequencer (Seikagaku Kogyo (Tokyo, Japan)). Iron atoms were quantified using an inductively coupled plasma mass spectrometer (VG Elemental, VG PQI). N-terminal sequence and amino acid composition analysis was performed with a 477A protein sequencer connected on line to a 120A PTH analyzer (Perkin-Elmer Applied Biosystems) and an 835 Amino Acid Analyzer (Hitachi), respectively.

Materials—TPCK-treated trypsin (type, XIII; from bovine pancreas), carboxypeptidase Y, leucine aminopeptidase M, L-cysteine-sulfonic acid, and N-acetylcysteine were purchased from Sigma, and thermolysin from Seikagaku Kogyo (Tokyo, Japan). N-Methyl-n-glucamine dithiocarbamate (MGD) was a generous gift from Dr. I. Ueno of RIKEN. 3-Nitrobenzyl alcohol, thioglycolic acid, and glycerol were the products of Tokyo Kasei (Tokyo, Japan), and 2,5-dihydroxybenzoic acid was purchased from Aldrich. All the other reagents used were of the highest biochemical grade available.

RESULTS

Proteolysis of the $\alpha$ Subunit of Inactive NHase—We first cleaved the $\alpha$ subunit with trypsin in the dark. Even after cleavage, the absorption peak at 370 nm, characteristic of the

\textsuperscript{4} J. A. Duine, personal communication.

inactive form, remained and disappeared upon light irradiation (data not shown). The excitation profile of the resonance Raman spectra of the inactive form suggested that this band was due to the transitions with both the iron $\leftrightarrow$ NO and iron $\leftrightarrow$ S charge transfer characters (9). To obtain the photoresponsive peptide, the digest was separated by reversed-phase HPLC on a column connected to a diode array detector. Since the absorbance at 370 nm readily disappeared both below pH 6 and above pH 9, the pH values of the solvents used in HPLC were controlled at 7.5. The chromatogram at 215 nm showed 13 peaks while that at 370 nm contained only one peak at 20.6 min (Fig. 1, A and B). Protein sequence analysis revealed that the 20.6-min peak contained a peptide fragment of 24-amino acids, Asn105-Val-Ile-Val-(Cys)-Ser-Leu-(Cys)-Ser-(Cys)-Thr-Ala-Trp-[\[(MGD)_{2}\text{-FeII-NO}] (NK24). This region is highly conserved among all known NHases and contains the Cys cluster (Cys109-Ser-Leu-Cys112) (NK24). This region is highly conserved among all known NHases and contains the Cys cluster (Cys109-Ser-Leu-Cys112). When the tryptic digest was irradiated prior to injection, the elution profile monitored at 215 nm was identical to that of the non-irradiated digest except that the 20.6-min peak delayed to 22.4 min (Fig. 1C). Since the amino acid sequences were identical, the shift would be induced by release of NO and/or iron. Fig. 2 shows the UV-Vis difference spectrum between the 22.4-min peak of the irradiated digest and the 20.6-min peak of the non-irradiated one. It exhibited two positive peaks at 280 and 370 nm, which is in good agreement with the photo-induced absorptive change of the isolated a subunit (21).

Detection of Iron Atoms and NO in NK24—We investigated the quantitative measurements of both the iron atom and NO in NK24 isolated from the inactive enzyme. The amount of iron atoms in NK24 was measured to be 0.79 iron/molecule with inductively coupled plasma mass spectrometry. NO molecules associated with NK24 were investigated with the use of a spin trap reagent, [\[(MGD)_{2}\text{-FeII}] alone. MGD and FeSO$_4$ was dissolved in buffer (20 mM Tris-HCl, pH 7.5, containing 2 mM 2-mercaptoethanol) at final concentrations of 75 and 15 mM, respectively. NK24 (20$\mu$M) in the same buffer, C, NK24 (20$\mu$M) in the presence of [\[(MGD)_{2}\text{-FeII}]. The concentration of MGD and FeSO$_4$ was 75 and 15 mM, respectively. All ESR spectra were measured in the dark with the same gain (2$\times$10$^5$) in a quartz cuvette (light pass, 0.3 mm) at room temperature. Instrument settings were as follows: modulation frequency and amplitude, 100 KHz and 0.1 millitesla, respectively; microwave power, 2.6$\mu$W.

was also ESR silent (Fig. 3B). However, when [\[(MGD)_{2}\text{-FeII}] was added to the solution in the dark, clear ESR signals of [\[(MGD)_{2}\text{-FeII-NO}] were observed (Fig. 3C), demonstrating that this peptide intrinsically possessed NO molecules. Unlike the native NHase in the inactive form, the ESR spectrum appeared without light stimulation. The reason for this discrepancy is unknown. However, it is probable that [\[(MGD)_{2}\text{-FeII}] attacks the nitrosyl iron in NK24 because of the absence of other protein moieties.

Isolation of the Nitrosylated Iron Center Complex—To obtain the minimum peptide segment required for the nitrosylated iron center, we attempted to digest NK24 with amino- and carboxypeptidases in the dark. At first, NK24 (450 pmol) was treated with thermolysin at a molar ratio of 1:10 so that it could be efficiently digested by exopeptidases. Subsequently, carboxypeptidase Y and leucine aminopeptidase M were added at a molar ratio of 1:50, and the reaction mixture was incubated for 12 h at 37 °C. The digest was analyzed by reversed-phase HPLC in pH 7.5 in the dark (Fig. 4). A peak at 11.6 min contained a peptide of 11 amino acids from Ile107 to Trp117 (IW11) while other peaks contained hydrolyzed amino acids and buffer components. The yield of IW11 was estimated to be 28%. The UV-Vis absorption spectrum of the 11.6-min peak exhibited the absorption peak at 370 nm (inset of Fig. 4), indicating that the nitrosyl iron was still associated with IW11. When NK24 was irradiated prior to digestion with thermolysin and exopeptidases, the 11.6-min peak completely disappeared (data not shown). Photodissociation of NO might destabilize the iron center, and induce the subsequent proteolysis of the peptide.
The spectrum was obtained by the same diode array detector as (2)). Thus, the 112th Cys residue of the active form of the NHase.  

Post-translational Modification of the Cys Residue in NK24—Fig. 5 shows FAB-MS spectrum of NK24 prepared from the photoactivated NHase (NK24 active). The iron atom was dissociated from NK24 by reversed-phase HPLC in the presence of trifluoroacetic acid. The molecular mass of NK24 active was m/z 2693.6, which is 30.3 Da larger than that deduced from the corresponding gene sequence. The molecular mass of the isolated α subunit was about 30 Da larger than the expected value by electrospray (data not shown). These cannot be attributed to nitrosothiolation of side chains of Cys residues by NO released from the iron center, because NO is quantitatively released into the solvent upon photoactivation (8) and the nitrosothiol group is unstable in the acidic solvent used in reversed-phase HPLC for isolation of the peptide. Thus, it was suggested that the NHase have a post-translational modification in this region of the α subunit.

Sequence analysis of NK24 active indicated that all residues were normal except three Cys residues, which are not stable in automated Edman degradation without alkylation of the sulfhydryl groups. Therefore, it was most probable that one or two Cys residues were modified to cause a mass increase of 30 Da. To identify the modified Cys residue(s), we carboxymethylated the Cys residues after reduction. Cys109 and Cys114 were identified as carboxymethylcysteines, whereas Cys112 remained undetectable. The molecular mass of reduced and carboxymethylated NK24 active was determined by MALDI-TOF MS to be 2837.3 Da, which well coincided with the expected value, 2837.3 Da, calculated mass with two carboxymethyl group introduced: 2779.3; B, recombinant α subunit expressed in E. coli after carboxymethylation with reduction (observed mass: (ME), calculated mass with three carboxymethyl group introduced: 2837.3).

To rule out the possibility that the modification is an artifact caused during the preparation of NK24, we analyzed the corresponding fragment prepared from the recombinant α subunit (αrec) expressed in E. coli. Like the native α subunit, αrec was purified by anion-exchange chromatography in the presence of 6 M urea. The purified αrec was colorless, indicating the absence of an iron center (data not shown). This αrec was readily cleaved with trypsin and carboxymethylated after reduction. The corresponding fragment was purified by reversed-phase HPLC and analyzed by sequence analysis and MALDI-TOF MS (Fig. 6B). All three Cys residues in the fragment were carboxymethylated and the molecular mass of the fragment was 2837.0 Da, which well coincided with the expected value, 2837.3 Da (the molecular mass calculated from the gene sequence (2663.3) plus that of three carboxymethyl groups (+59 × 3 – H × 3)). Therefore, the modification of the 112th Cys residue seems to be specific to Rhodococcus sp. N-771.

Identification of the Post-translational Modification of the 112th Cys Residue—Finally, we studied the structure of the modified Cys112. The molecular mass increment of 32 Da can be attributed to nitrosothiolation of side chains of Cys residues by NO released from the iron center, because NO is quantitatively released into the solvent upon photoactivation (8) and the nitrosothiol group is unstable in the acidic solvent used in reversed-phase HPLC for isolation of the peptide. Thus, it was suggested that the NHase have a post-translational modification in this region of the α subunit.

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Identification of the Post-translational Modification of the 112th Cys Residue—Finally, we studied the structure of the modified Cys112. The molecular mass increment of 32 Da can be likely explained by derivatization of the 112th Cys residue by one sulfur atom (Cys-SH) or two oxygen atoms (cysteine-sulfinic acid (Cys-SO2H); Fig. 7). Since Cys-S-SH should be carboxymethylated after reduction, it was assumed that Cys112 was modified to Cys-SO2H. We subjected the peptide to amino acid analysis through derivatization with FLEC (23). Avoiding oxidation during hydrolysis in acidic conditions, NK24 active was...
hydrolyzed enzymatically. We prepared NK24active from the irradiated α subunit whose sulphydryl groups were blocked by carboxymethylation after reduction in advance. Since Cys112 is the eighth amino acid residue from the N terminus of the peptide, we further cleaved the peptide with thermolysin prior to exopeptidase hydrolysis. The resulting octapeptide, LP8active (Leu111-X-Ser-X-Cys-Thr-Trp-Pro118, *Cys designates carboxymethylcysteine), was purified by reversed-phase HPLC. The molecular mass of the octapeptide estimated by MALDI-TOF MS was m/z 970.7, 32.6 Da larger than that calculated from the gene sequence. The chromatograms of amino acid analysis of the authentic L-Cys-SO3H and L-Cys-SO2H after FLEC treatment are shown in Fig. 8, A and B. FLEC derivatives of L-Cys-SO3H, d-Cys-SO3H, and L-Cys-SO2H (FLEC-L-Cys-SO3H, FLEC-d-Cys-SO3H, and FLEC-l-Cys-SO2H) were eluted at 22.9, 23.4, and 26.7 min, respectively. About 40% of the authentic L-Cys-SO2H was oxidized to l-Cys-SO3H. When LP8active, hydrolyzed with leucine aminopeptidase M was submitted to amino acid analysis after FLEC treatment, the peaks of FLEC-L-Cys-SO3H as well as FLEC-l-Cys-SO2H were observed (Fig. 8C). FLEC derivatives of other amino acids were eluted after 43 min (data not shown). The amount of FLEC-L-Cys-SO3H and FLEC-l-Cys-SO2H were 44 and 55 pmol, respectively. The yield of the Leu residue at the N terminus was estimated to be 97.3 pmol, thus the octapeptide contained one L-Cys-SO2H per molecule. From these results, we concluded that the 112th Cys residue of the α subunit is post-translationally oxidized to a sulfonic acid derivative in the intact NHase from *Rhodococcus* sp. N-771.

**DISCUSSION**

Cysteine-Sulfonic Acid Stabilization in the Iron Center of NHase—Mass spectrometry is a useful technique for identification of a post-translational modification in a protein (27). Using this technique, we have shown that Cys112 stably exists as a sulfonic acid derivative in the intact NHase. It is unlikely that the derivatization is an artifact because Cys112 is specifically oxidized among the Cys-cluster (Fig. 6A) and because the recombinant α subunit expressed in *E. coli* (αrec) contains no derived Cys residues despite almost the same purification procedures (Fig. 6B). It is unknown how aCys112 is post-translationally modified to Cys-SO3H. NO molecules might oxidize aCys112 in *Rhodococcus* sp. N-771. There are several reports describing that NO and ONOO−, which is produced from O2 and NO, indeed oxidize protein sulphydryls (28, 29). Unfortunately, αrec does not reconstitute the NHase with the β subunit (data not shown) and, therefore, we cannot investigate whether Cys-SO2H is essential for the photoactivation and/or the catalysis. The inability to reconstitute the NHase is not due solely to the absence of the Cys-SO2H derivatization, because the α subunit isolated from the active form also does not reassemble with the β subunit (21). The sulfonic acid derivative is stabilized in the native inactive NHase and does not undergo further oxidation even under aerobic conditions as long as it is kept in the dark, strongly suggesting that the Cys112 residue coordinates to the iron atom. The coordinated atom is the sulfur atom or most likely one of the oxygen atoms in the sulfonic acid group because the donation effect of the sulfur atom must be significantly weakened by two bound oxygen atoms.

Another example of a protein containing Cys-SO3H is SP-22, an unidentified substrate protein for mitochondrial ATP-dependent protease in bovine adrenal cortex (30). SP-22 is a homologomer consisting of M21,600 subunits, and the 47th amino acid of which was found to be a sulfonic acid by FAB-mass spectrometry. The fact that the vicinal sequence is highly conserved among several homologous proteins suggests that this residue is important to the biological function of SP-22. It remains possible that this residue exists as a cysteine-sulfenic acid (Cys-SOH) or a normal cysteine in the native SP-22 protein. On the other hand, it has been shown that two flavoproteins, NADH oxidase and NADH peroxidase from *Enterooccus faecalis* 10C1, possess Cys-SOH residues at non-flavin redox centers in the native enzymes (31, 32). These modified Cys residues play an essential role in the catalysis, being switched between Cys-SOH and Cys-S− during turnover (31). Furthermore, Cys-SOH/Cys-SH redox cycle may be important for DNA binding activity of transcription factors including E2 protein (33), OxyR (34, 35), Jun and Fos (36), and nuclear factor I (37). However, none of these proteins are metalloproteins and the amino acid sequences around the modified Cys residues show no homology with the NHase. Therefore, the role of Cys-SO3H in NHase may be unique.

**Structure of the Iron Center of the NHase in the Inactive Form—**Cys109 and Cys114 of NK24 prepared from the inactive form were not modified by carboxymethylation even after reduction (data not shown), whereas those from the active form were carboxymethylated by the same treatment (Fig. 6A). Thus, the sulphydryl groups of these Cys residues are stabilized by coordination to the nitrosyl-iron. The results of the present study suggested that, in the inactive state, the ligands in the six-coordinate sphere of the non-heme iron center include two cysteine thiolates, and one oxygen atom of sulfonic acid and one NO molecule. A model of the non-heme iron center of the NHase in the inactive form is illustrated in Fig. 9.

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**Fig. 7. Structure of cysteine-sulfonic acid.**

**Fig. 8. Chromatograms of amino acid analysis of LP8active, using FLEC derivatization.** A, FLEC derivatives of dL-cysteic acid (Cys-SO3H), 100 pmol; B, FLEC derivative of dL-cysteine-sulfonic acid (Cys-SO2H), 100 pmol; C, LP8 hydrolyzed with leucine aminopeptidase M and derived with FLEC, 100 pmol. *C designates carboxymethylcysteine.* The elution conditions were described under “Experimental Procedures.” Elution was monitored by fluorescence with excitation and emission wavelengths of 260 and 315 nm, respectively.
two ligands remain unknown. Previous spectroscopic studies of the active enzyme suggested the coordination of His imidazole ligands at these sites (14, 20). However, very recently the crystal structure of the *Rhodococcus* R312 NHase in the active form was unveiled and it was revealed that, in the active form, the ligands to the iron are the side chains of αCyγ109, αCyγ113, and αCy8115 (corresponding to αCyγ109, αCyγ112, and αCyγ114 of the *Rhodococcus* N-771 enzyme),5 and the main chain amide nitrogens of αSe114 and αCyγ115 (corresponding to αSe114 and αCyγ114 of the *Rhodococcus* N-771 enzyme) (38). Consistently with the crystal structure, the fact that IW11, a minimum peptide segment, does not contain a His residue as well as potential side chain nitrogens of the active enzyme suggested the coordination of His imidazole nitrogens to the metal site structure of the active form with respect to the modification of a Cys-SO2H in the metalloprotein. The biological role of a Cys-SO2H in the native NHase. The biological role of Cys-SO2H in the photoresponse and/or catalysis is still unclear. The structure of the *Rhodococcus* N-771 enzyme. The reason for this discrepancy remains unclear. The structure of the *Rhodococcus* N-771 enzyme may be different from that of the *Rhodococcus* R312 enzyme. X-ray crystallographic analysis of the inactive form of the *Rhodococcus* N-771 enzyme is currently underway in our laboratory (39). There is no data to suggest the iron center bonds with an oxygen atom of Cys-SO2H except for the results of this study. Model complexes having similar iron coordination spheres should be investigated. Also, it should be clarified whether the corresponding Cys residues of other iron- and cobalt-containing NHases are modified by sulfonic acid derivatives.

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5 The Met residue at the N terminus is co-translationally removed in the α subunit of the *Rhodococcus* N-771 enzyme.