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A Novel Inhibitor for Fe-type Nitrile Hydratase: 2-Cyano-2-propyl Hydroperoxide

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Abstract: Nitrile hydratase (NHase) is a non-heme iron or non-corrin cobalt enzyme having two post-translationally modified ligand residues, cysteine-sulfinic acid (αCys112-SO_2H) and -sulfenic acid (αCys114-SOH). We studied the interaction between Fe-type NHase and isobutyronitrile (*iso*-BN) which had been reported as a competitive inhibitor with a K_i value of 5 μM. From detailed kinetic studies of the inhibitory effect of *iso*-BN on Fe-type NHase, we found that authentic *iso*-BN was hydrated normally and that the impurity present in commercially available *iso*-BN inhibited NHase activity strongly. The inhibitory compound induced significant changes in the UV−vis absorption spectrum of NHase, suggesting its interaction with the iron center. This compound was purified by using reversed-phase HPLC and identified as 2-cyano-2-propyl hydroperoxide (Cpx) by ^{1}H and PFG-HMBC NMR spectroscopy. Upon addition of a stoichiometric amount of Cpx, NHase was irreversibly inactivated, probably by the oxidation of αCys114-SOH to Cys-SO_2H. This result suggests that the −SOH structure of αCys114 is essential for the catalytic activity. The oxygen atom in Cys-SO_2H is confirmed to come from the solvent H_2O. The oxidized NHase was found to induce the UV−vis absorption spectral changes by addition of Cpx, suggesting that Cpx strongly interacted with iron(III) in the oxidized NHase to form a stable complex. Thus, Cpx functions as a novel irreversible inhibitor for NHase.

Introduction

Nitrile hydratase (NHase, EC 4.2.1.84)[1] is a bacterial metalloenzyme having a low spin non-heme iron(III) or a non-corrin cobalt(III) at the catalytic center and grouped into the Fe-type and the Co-type NHase families.[2−4] The enzyme consists of α- and β-subunits, each with an Mr of about 23k and generally exists as $\alpha\beta$M or $(\alpha\beta M)_2$ structures.[5,6] NHase catalyzes the hydration of nitriles to the corresponding amides and is involved in the assimilation of nitriles in various microorganisms such as *Rhodococcus*, *Pesudomonas*, and related bacteria. NHase is industrially important in the production of acrylamide and nicotinamide.[2−4] Currently, more than 30 000 tons of acrylamide is produced by using this enzyme. Significant amino acid sequence homology[7−13] and the similarities of ESR[14,15] and X-ray absorption[16,17] spectra between the Fe- and Co-type

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(1) Asano, Y.; Tani, Y.; Yamada, H. *Agric. Biol. Chem.* **1980**, *44*, 2251−2252.
(2) Kobayashi, M.; Nagasawa, T.; Yamada, H. *Trends Biotechnol.* **1992**, *10*, 402−408.
(3) Yamada, H.; Kobayashi, M. *Biosci. Biotechnol. Biochem.* **1996**, *60*, 1391−140.
(4) Kobayashi, M.; Shimizu, S. *Nat. Biotechnol.* **1998**, *16*, 733−773
(5) Odaka, M.; Noguchi, T.; Nagashima, S.; Yohda, M.; Yabuki, S.; Hoshino, M.; Inoue, Y.; Endo, I. *Biochem. Biophys. Res. Commun.* **1996**, *221*, 146−150.
(6) Nakasako, M.; Odaka, M.; Yohda, M.; Dohmae, N.; Takio, K.; Kamiya, N.; Endo, I. *Biochemistry* **1999**, *38*, 9887−9898.
(7) Nojiri, M.; Yohda, M.; Odaka, M.; Matsushita, Y.; Tsujimura, M.; Yoshida, T.; Dohmae, N.; Takio, K.; Endo, I. *J. Biochem. (Tokyo)* **1999**, *125*, 696−704.
(8) Ikehata, O.; Nishiyama, M.; Horinouchi, S.; Beppu, T. *Eur. J. Biochem.* **1989** *181*, 563−570.
(9) Mayaux, J.; Cerbelaud, E.; Soubrier, F.; Faucher, D.; Pétré, D. *J. Bacteriol.* **1990**, *172*, 6764−6773.
(10) Nishiyama, M.; Horinouchi, S.; Kobayashi, M.; Nagasawa, T.; Yamada, H.; Beppu, T. *J. Bacteriol.* **1991**, *173*, 2465−2472.
(11) Kobayashi, M.; Nishiyama, M.; Nagasawa, T.; Horinouchi, S.; Beppu, T.; Yamada, H. *Biochim. Biophys. Acta* **1991**, *1129*, 23−33.
(12) Payne, M. S.; Wu, S.; Fallon, R. D.; Tudor, G.; Stieglitz, B.; Turner, I. M., Jr.; Nelson, M. J. *Biochemistry* **1997**, *36*, 5447−5454.
(13) Katayama, Y.; Matsushita, Y.; Kaneko, M.; Kondo, M.; Mizuno, T.; Nyunoya, H. *J. Bacteriol.* **1998**, *180*, 2583−2589.
(14) Sugiura, Y.; Kuwahara, J.; Nagasawa, T.; Yamada, H. *J. Am. Chem. Soc.* **1987**, *109*, 5848−5850.
(15) Brennan, B. A.; Alms, G.; Nelson, M. J.; Durney, L. T.; Scarrow, R. C. *J. Am. Chem. Soc.* **1996**, *118*, 9144−9145.

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NHase families suggest that the structures and catalytic mechanisms are conserved between the two types of NHases. Unlike Co-type NHases, the catalytic activity of the Fe-type enzymes is regulated by nitrosylation and photoinduced denitrosylation of the iron centers.[18−21]

Recently, the crystal structures of Fe-type as well as Co-type NHases were elucidated.[6,22−24] The first structure is that of Fe-type NHase from *Rhodococcus* sp. R312 in the denitrosylated state.[22] All the ligand residues were located in the highly conserved sequence motif in the α-subunit, Cys1-Ser1-Leu-Cys2-Ser2-Cys3, where Ser1 was replaced by Thr in Co-type NHases. The non-heme iron was coordinated to two main chain amide nitrogen atoms of Ser2 and Cys3 and to three sulfur atoms of Cys1−3. The sixth ligand was not identified probably due to the limit of resolution. Then, we determined the structure of Fe-type NHase of *Rhodococcus* sp. N771 in the nitrosylated state.[23] Nucleotide sequences of the genes encoding the NHases of *Rhodococcus* sp. R312 and sp. N771 showed that they are identical.[7,9] This nitrosylated structure was more precise and revealed that αCys112 (corresponding to Cys2) and αCys114 (corresponding to Cys3) were modified post-translationally to cysteine-sulfinic (Cys-SO_2H) and -sulfenic acid (Cys-SOH), respectively. These modifications were also identified by mass spectrometry (MS).[23,25] NHase is the first protein having both Cys-SO_2H and Cys-SOH as metal ligands. Very recently, elucidation of the crystal structure of the Co-type NHase of *Pseudonocardia thermophilia* JCM 3095[24] demonstrated the structural conservation of the metallocenters including both post-translational modifications among Fe-type and Co-type NHase families.

Some mechanistic studies of nitrile hydration by these enzymes have focused on the function of the modified cysteine residues. The NHase reconstituted from the recombinant unmodified α and β subunits showed no catalytic activity but acquired the activity after the aerobic oxidation of αCys112 to Cys-SO_2H.[26] Also, the Co-substituted mutant of Fe-type NHase of *Rhodococcus* sp. N771 was activated by oxidation with potassium hexacyanoferrate.[27] A number of Fe(III) and Co(III) model complexes mimicking the metallocenters of NHase have been synthesized and characterized.[28−34] Heinrich et al. succeeded in synthesizing the disulfenato compound, Na[Co-($L-N_2SOSO)_2$], and demonstrated that the sulfenato group of the compound was necessary for the nitrile hydration activity.[34] Thus, the post-translational modifications of αCys112 and αCys114 were essential to the catalytic reaction of NHase. However, the catalytic mechanism of NHase remains unknown including its substrate recognition and binding.

One of the best ways to study the catalytic mechanism of an enzyme is to use inhibitors and/or substrate analogues. However, in the case of NHase, little information is available on inhibitors which are analogous to the substrates. Generally speaking, NHases exhibit wide substrate specificity.[35−41] It is of interest that isobutyronitrile ($(CH_3)_2CHCN$, *iso*-BN) was barely hydrated and functioned as a competitive inhibitor of Fe-type NHases from *Rhodococcus* sp. R312[35] and *Pseudomonas chlororaphis* B23[36] with K_i values of 5.4 and 3.5 μM. The K_i values are rather small considering that NHase hydrates other nitriles with K_m values of 10^2-10^3 μM order. In addition, these findings are curious because *n*-butyronitrile is normally hydrated by these enzymes[35,36] and because some other Fe-type NHases hydrate it with a relatively low rate constant.[37−41] Thus, we have studied the interaction between *iso*-BN and Fe-type NHase in detail. We found that commercially available *iso*-BN reagent contained a compound that associated specifically with NHase to inactivate it. Here, we show a novel inhibitor, 2-cyano-2-propyl hydroperoxide ($(CH_3)_2-C(CN)-OOH$), for Fe-type NHase.

Experimental Section

Materials. *iso*-BN was purchased from Wako Pure Chemical Industries (Japan). TPCK-treated trypsin (type XIII: from bovine pancreas) was the product of Sigma−Aldrich (USA). Stable isotope $H_2{}^{18}O$ (95%) was purchased from Shoko Co., Ltd (Japan). 2-Cyano-2-propyl hydroperoxide (Cpx) was synthesized according to the method of Dulog and Vogt with slight modification.[42] All the other reagents used in this study were of the highest grade available.

Preparation of NHase. NHase of *Rhodococcus* sp. N771 was nitrosylated in vivo and then purified in the dark, as described previously.[43] The purified NHase was stored in the nitrosylated state in a 60% saturated ammonium sulfate suspension in the dark. Prior to experiments, the nitrosylated NHase was dissolved in buffer A (50 mM

(16) Nelson, M. J.; Jin, H.; Turner, I. M., Jr.; Grove, G.; Scarrow, R. C.; Brennan, B. A.; Que, L., Jr. *J. Am. Chem. Soc.* **1991**, *113*, 7072−7073.
(17) Scarrow, R. C.; Brennan, B. A.; Cummings, J. G.; Jin, H.; Duong, D. J.; Kindt, J. T.; Nelson, M. J. *Biochemistry* **1996**, *35*, 10078−10088.
(18) Noguchi, T.; Honda, J.; Nagamune, T.; Sasabe, H.; Inoue, Y.; Endo, I. *FEBS Lett.* **1995**, *358*, 9−12.
(19) Odaka, M.; Fujii, K.; Hoshino, M.; Noguchi, T.; Tsujimura, M.; Nagashima, S.; Yohda, M.; Nagamune, T.; Inoue, Y.; Endo, I. *J. Am. Chem. Soc.* **1997**, *119*, 3785−3791.
(20) Noguchi, T.; Hoshimo, M.; Tsujimura, M.; Odaka, M.; Inoue, Y.; Endo, I. *Biochemistry* **1996**, *35*, 16777−16781.
(21) Scarrow, R. C.; Strickler, B. S.; Ellison, J. J.; Shoner, A. C.; Kobacs, J. A.; Cummings, J. G.; Nelson, M. J. *J. Am. Chem. Soc.*, **1998**, *120*, 9237−9245.
(22) Huang, W.; Jia, J.; Cummings, J.; Nelson, M. J.; Schneider, G.; Lindqvist, Y. *Structure* **1997**, *5*, 691−699.
(23) Nagashima, S.; Nakasako, M.; Dohmae, N.; Tsujimura, M.; Takio, K.; Odaka, M.; Yohda, M.; Kamiya, N.; Endo, I. *Nat. Struct. Biol.* **1998**, *5*, 347−351.
(24) Miyanaga, A.; Fushinobu, S.; Ito, K.; Wakagi, T. *Biochem. Biophys. Res. Commun.* **2001**, *288*, 1169−1174.
(25) Tsujimura, M.; Dohmae, N.; Odaka, M.; Chijimatsu, M.; Takio, K.; Yohda, M.; Hoshino, M.; Nagashima, S.; Endo, I. *J. Biol. Chem.* **1997**, *272*, 29454−29459.
(26) Murakami, T.; Nojiri, M.; Nakayama, H.; Odaka, M.; Yohda, M.; Dohmae, N.; Takio, K.; Nagamune, T.; Endo, I. *Protein Sci.* **2000**, *9*, 1024−1030.
(27) Nojiri, M.; Nakayama, H.; Odaka, M.; Yohda, M.; Takio, K.; Endo, I. *FEBS Lett.* **2000**, *465*, 173−177.
(28) Mascharak, P. K. *Coord. Chem. Rev.* **2002**, *225*, 201−214.
(29) Beissel, T.; Bruger, K. S.; Voigt, G.; Wieghardt, K.; Butzlaff, C.; Trautwein, A. X. *Inorg. Chem.* **1993**, *32*, 124−126.
(30) Noveron, J. C.; Olmstead, M. M.; Mascharak, P. K. *J. Am. Chem. Soc.* **2001**, *123*, 3247−3259.
(31) Artaud, I.; Chatel, S.; Chauvin, A. S.; Bonnet, D.; Kopf, M. A.; Leduc, P. *Coord. Chem. Rev.* **1999**, *192*, 577−586.
(32) Schweitzer, D.; Shearer, J.; Rittenberg, D. K.; Shoner, S. C.; Ellison, J. J.; Loloee, R.; Lovell, S.; Barnhart, D.; Kobacs, J. A. *Inorg. Chem.* **2002**, *41*, 3128−3136.
(33) Shearer, J.; Jackson, H. L.; Schweitzer, D.; Rittenberg, D. K.; Leavy T. M.; Kaminsky, W.; Scharrow, R. C.; Kobacs, J. A. *J Am. Chem. Soc.* **2002**, *124*, 11417−11428.
(34) Heinrich, L.; Mary-Verla, A.; Li, Y.; Vaissermann, J.; Chottard, J. C. *Eur. J. Inorg. Chem.* **2001**, *9*, 2203−2206.
(35) Nagasawa, T.; Ryuno, K.; Yamada, H. *Biochem. Biophys. Res. Commun.* **1986**, *139*, 1305−1312.
(36) Nagasawa, T.; Nanba, H.; Ryuno, K.; Takeuchi, K.; Yamada, H. *Eur. J. Biochem.* **1987**, *162*, 691−698.
(37) Watanabe, I.; Satoh, Y.; Enomoto, K. *Agric. Biol. Chem.* **1987**, *51*, 3193−3199.
(38) Tani, Y.; Kurihara, M.; Nishie, H. *Agric. Biol. Chem.* **1989**, *53*, 3151−3158.
(39) Nagasawa, T.; Takeuchi, K.; Yamada, H. *Eur. J. Biochem.* **1991**, *196*, 581−589.
(40) Duran, R.; Nishiyama, M.; Horinouchi S.; Beppu, T. *Biosci. Biotechnol. Biochem.* **1993**, *57*, 1323−1328.
(41) Cramp, R. A.; Cowan, D. A. *Biochim. Biophys. Acta* **1999**, *1431*, 249−260.
(42) Dulog, L.; Vogt, W. *Tetrahedron Lett.* **1966**, *42*, 5169−5174.
(43) Tsujimura, M.; Odaka, M.; Nagashima, S.; Yohda, M.; Endo, I. *J. Biochem (Tokyo)* **1996**, *119*, 407−413.

Tris-HCl, pH 7.5, containing 40 mM *n*-butyric acid), desalted using Centriprep-30, and then activated by irradiation with 5000 lx of white light (71.0 W/m^2) from a photoreflector lamp (500 W SPOT, Toshiba, Japan) for 15 min in an ice bath. The concentration of NHase was determined by measuring the absorbance at 280 nm (ϵ_{280} = 1.5 mL mg^{-1} cm^{-1}) after photodissociation of nitric monooxide. For the oxidation of αCys114-SOH to Cys-SO_2H (NHase$_{(dSO2H)}$), the photodenitrosylated NHase was incubated in buffer B (50 mM Tris-HCl, pH 8.5) aerobically at 4 °C for 1 week. The oxidation of αCys114-SOH to Cys-SO_2H was confirmed by mass spectrometry (MS).[26] The characterization of the oxidized NHase will be reported elsewhere.[44]

Kinetic Analyses of NHase Activity. The catalytic activity of NHase was determined by measuring the initial rate of nitrile hydration using methacrylonitrile as a substrate.[7] The reaction was started by adding NHase to the reaction mixture. The initial rate was determined by continuously monitoring the absorbance of the product, methacrylamide, at 224 nm with ϵ_{224} = 2.5 mM^{-1} cm^{-1}. Assays were performed in 2.0 mL of 50 mM sodium phosphate, pH 7.5, containing the appropriate amounts of methacrylonitrile using a V-570 UV/vis/NIR spectrophotometer (JASCO Co., Ltd, Japan). One unit of the activity was defined as the quantity of NHase that produces 1 μmol of methacrylamide per minute. The inhibitory effect of *iso*-BN on NHase activity was examined by Dixon plot.[45] The kinetic analysis of the distilled *iso*-BN was performed according to the method mentioned above. The absorption coefficient of the hydration product, isobutylamide, at 220 nm was ϵ_{220} = 8.0 × 10^{-2} mM^{-1} cm^{-1}.

Isolation of the Inhibitory Compound from Commercially Available *iso*-BN. A 500 mL volume of commercially available *iso*-BN was distilled with a rotary evaporator at 27 °C until the volume of the residuum was reduced to ca. 20 mL. After the complete removal of *iso*-BN using a centrifugal concentrator, the residual substance was extracted with water. The extract was separated by reversed-phase HPLC on a Cosmosil 5C18-AR column (4.6 × 150 mm; Nacalai tesque, Japan) using a Gulliver 1500 Intelligent HPLC system (JASCO Co., Ltd, Japan). Solvent A was 0.09% (v/v) trifluoroacetic acid (TFA), and solvent B was 80% (v/v) acetonitrile containing 0.075% (v/v) TFA. The column was equilibrated with 2% solvent B and eluted by a linear gradient to 50% solvent B for 20 min at a flow rate of 0.5 mL/min.

NMR Analysis. NMR spectra were recorded on a JEOL JNM-A600 spectrometer (Japan) at 298 K. Chemical shifts were reported in parts per million (ppm). For ^{1}H NMR spectra, TMS was used as the internal reference at 0 ppm. For ^{13}C NMR spectra, the central peak of solvent $CDCl_3$ was applied as the reference. In 2D PFG-HMQC[46] and -HMBC[47] experiments, the matrix size was 2048 data points in the F_2 (^{1}H) frequency domain and 512 data points in the F_1 (^{13}C) frequency domain. PFG-HMBC spectra were recorded by 60 ms duration for long-range coupling with a *z*-axis pulsed field gradient.

MS of the Tryptic Peptide of NHase. NHase samples were precipitated by the addition of 10% (w/v) trichloroacetic acid (TCA) on ice for 30 min. A precipitate was collected by centrifugation at 15 krpm for 15 min at 4 °C and dissolved in 0.5 M Tris-HCl, pH 8.0, containing 8 M urea. The protein was reduced with 30 mM dithiothreitol for 1 h at 37 °C and carboxymethylated with 60 mM iodoacetic acid at room temperature in the dark for 30 min. The sample was diluted with three volumes of water, and treated with TPCK-treated trypsin (1/30 (w/w) of NHase) at 37 °C for 3 h. The tryptic digest was separated by reversed-phase HPLC at a flow rate of 30 μL/min on a Mightysil C8 column (2.0 × 50 mm, Kanto Kagaku, Japan), connected on an Agilent 1100 series liquid chromatograph (USA). Solvent A was 0.09% (v/v) TFA, and solvent B was 80% (v/v) acetonitrile containing 0.075% (v/v) TFA. The column was equilibrated with 2% solvent B, and the digest was eluted by a linear gradient to 99% solvent B for 20 min. The eluate was directly analyzed by a Finnigan LCQ ion trap mass spectrometer (USA) with an electrospray ionization probe. For the ^{18}O-labeling experiments, the tryptic peptide containing the iron binding site was fractionated by reversed-phase HPLC as described above and then analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) using a Reflex (Bruker, Germany) equipped with a delayed extraction ion source. Samples were prepared by mixing 0.5 μL of the sample dissolved in 33% (v/v) acetonitrile containing 0.07% (v/v) TFA with 0.5 μL of the 10 mg/mL 2,5-dihydroxybenzoic acid matrix solution on the target. Positive ion mass spectra were measured in the reflectron mode at an accelerating voltage of 28.5 kV. A mass scale was calibrated by using commercially available peptides.

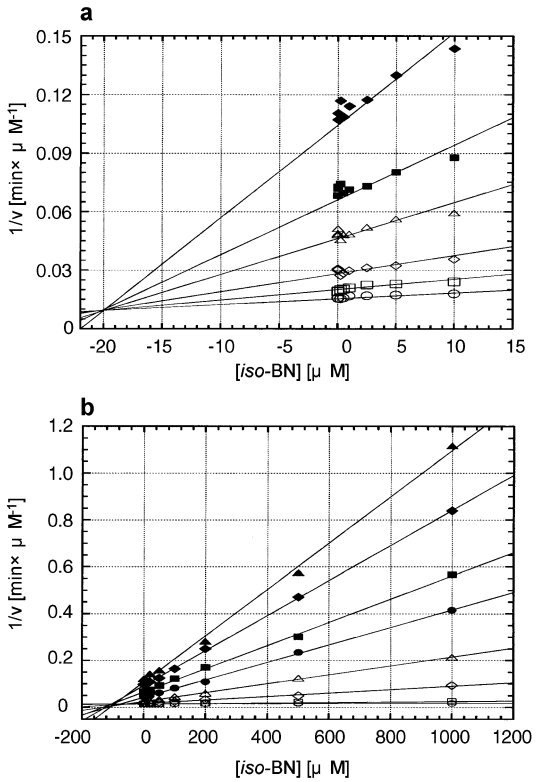

Figure 1. Inhibitory effect of *iso*-BN on NHase activity. Dixon plots showing the reciprocal values of the rate of methacrylonitrile hydration as a function of *iso*-BN concentration. (a) Commercially available *iso*-BN. Methacrylonitrile concentrations used were 0.070, 0.10, 0.17, 0.25, 1.0, and 20 mM, respectively. (b) Distilled *iso*-BN. Methacrylonitrile concentrations used were 0.050, 0.070, 0.10, 0.17, 0.40, 1.0, 5.0, and 20 mM, respectively.

^{18}O-Labeling Experiment. NHase was dissolved in 50 mM Tris-HCl, pH 7.5, containing $H_2{}^{18}O$ (93 atom % ^{18}O) to give a concentration of 44 μM. Cpx (2 equiv) was added to NHase (100 μL) and incubated for 30 min in an ice bath. The final concentration of $H_2{}^{18}O$ was 81 atom % ^{18}O. After the removal of $H_2{}^{18}O$ with centriprep-30 (Amicon), the sample was precipitated by the addition of 10% (w/v) TCA on ice for 30 min.

Results

Inhibition of NHase by *iso*-BN. The inhibitory effect of a commercially available *iso*-BN reagent on Fe-type NHase from *Rhodococcus* sp. N771 was studied by Dixon plot[45] (Figure 1A). The *iso*-BN reagent behaved as a strong competitive inhibitor for the enzyme with a K_i value of 20 μM, which was about 4 times larger than those reported previously.[35,36] To clarify the difference in K_i values, we distilled the reagent by a rotary evaporator and examined the inhibitory effect of the distilled

(44) Odaka, M.; Watanabe, N.; Nakayama, H.; Kawano, Y.; Kamiya, N.; Takio, K.; Endo, I. Manuscript in preparation.
(45) Dixon, M. *Biochem. J.* **1953**, *55*, 170−171.
(46) Hurd, R. E.; John, B. K. *J. Magn. Reson.* **1991**, *91*, 648−653.
(47) Wilker, W.; Leibfritz, D.; Kerssebaum, R.; Bermel, W. *Magn. Reson. Chem.* **1993**, *31*, 287−292.

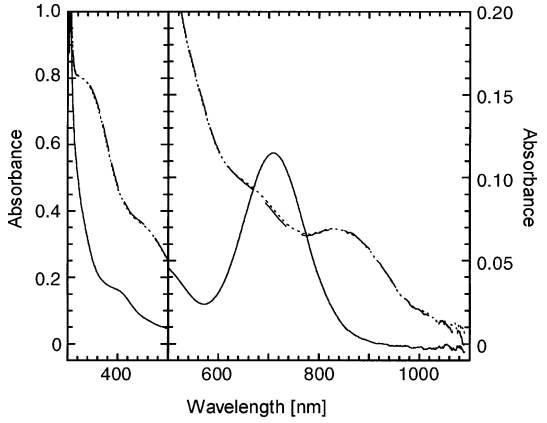

Figure 2. Effect of *iso*-BN on the UV−vis absorption spectra of NHase. Solid line, native NHase; dashed line, NHase in the presence of commercially available *iso*-BN (110 mM); dotted line, NHase in the presence of 1% (v/v) distillation residuum of commercially available *iso*-BN. NHase was dissolved in buffer A at a concentration of 100 μM. All spectra were recorded at room temperature with a V-570 UV/vis/NIR spectrophotometer (JASCO Co., Ltd, Japan).

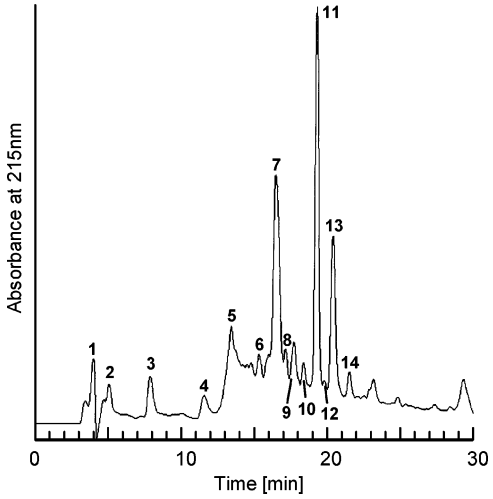

Figure 3. Isolation of the compound(s) from the distillation residuum of commercially available *iso*-BN by reversed-phase HPLC. The elution conditions were described in the Experimental Section.

iso-BN on NHase activity. Unexpectedly, the obtained K_i value was 100 μM (Figure 1B), 5 times larger than that of commercially available *iso*-BN. However, 1% (v/v) of the residuum of distillation inhibited completely NHase activity. The distilled *iso*-BN was hydrated with the kinetic parameters; $K_m = 4.7$ mM and $V_{max} = 1.0 \times 10^3$ units/mg protein, whereas only a trace amount of commercially available *iso*-BN was hydrated by the same amount of NHase. These results clearly indicated that the strong competitive inhibitory effect of the commercially available *iso*-BN reagent was caused not by authentic *iso*-BN but by one or more compounds contaminating the reagent. When the fact that the purity of the commercially available *iso*-BN reagent used in these experiments was guaranteed at more than 97% is considered, the compound(s) should interact with NHase with extreme high affinity.

Interaction between NHase and the Compound(s). We measured the UV−vis absorption spectra of NHase in the presence of commercially available *iso*-BN and the distillation residuum. In the absence of the *iso*-BN reagent, NHase shows a characteristic peak at around 711 nm with a shoulder peak around 395 nm (Figure 2, solid line). These peaks have been assigned as the S → Fe^{III} charge-transfer band in Fe-type NHases.[20,48] When the commercially available *iso*-BN (Figure 2, dashed line) or the distillation residuum (Figure 2, dotted line) was added to NHase, these peaks disappeared and, in turn, a new peak appeared at 830 nm with shoulder peaks at about 340, 440, and 660 nm. These results suggested that the compound(s) was(were) directly associated with the iron center. Hereafter, we attempted to identify the compound(s).

Isolation of the Compound(s) from Commercially Available *iso*-BN Reagent. The distillation residuum of commercially available *iso*-BN was separated by reversed-phase HPLC as described in the Experimental Section. Fourteen major peaks detected in the elution profile at 215 nm (Figure 3) were collected. The effect of each fraction on the visible absorption spectra of NHase was examined. Only the fraction of peak number 7 induced spectral changes similar to those observed in the presence of the distillation residuum (data not shown). Consistent with the visible spectral changes, NHase activity was lost only on the addition of peak fraction 7. Thus, the compound that specifically inhibited NHase was likely to be a single substance present in fraction 7.

Identification of the Compound. To identify the compound chemically, the fraction of peak 7 was analyzed by ^{1}H NMR and PFG-HMBC NMR spectroscopy. In the ^{1}H NMR spectrum of the compound, two singlet signals were observed at 1.64 (6H) ppm and 8.62 (ca. 1H) ppm. In the PFG-HMBC spectrum, the methyl singlet signal at 1.64 ppm had long-range correlations to the carbons at 24.4, 77.0, and 120.0 ppm. The ^{13}C chemical shifts suggested the presence of 2 equiv of methyl groups, an oxygenated quaternary carbon, and a nitrile group. The speculated functional groups and ^{13}C NMR spectral data were similar to those of acetone cyanohydrin ($(CH_3)_2C(OH)CN$). A characteristic ^{1}H NMR signal of the isolated compound was the broad singlet signal at 8.62 ppm that suggested the presence of a hydroperoxy group. Based on the NMR spectral data mentioned above, the structure of the isolated compound was speculated to be 2-cyano-2-propyl hydroperoxide (2-hydroperoxy-2-methylpropionitrile, $(CH_3)_2C(OOH)CN$; Cpx). The NMR data of the authentic Cpx[42] were completely identical to those of the isolated compound. From this experimental evidence, the isolated compound was identified as Cpx.

Inactivation of NHase by Authentic Cpx. The catalytic activity of NHase was measured after incubation with authentic Cpx at various concentrations for 30 min (Figure 4, open circle). NHase activity decreased along with the increase in the Cpx concentration: ca. 2% of the original activity at a molar ratio of 1:1 (NHase:Cpx). The NHase activity never recovered even after a thorough dialysis against buffer A. Since Cpx was likely to oxidize the post-translationally modified ligand residues, αCys112-SO_2H and αCys114-SOH, the tryptic peptide containing these residues, ^{105}NVIVCSLCSCTAWPILGLPPTWY^{128}K (NK24), isolated from the Cpx-treated NHase was studied by MS. The *m*/*z* 1407 peak corresponded to the $[M + 2H]^{2+}$ ion of NK24 of native NHase, and the 1394 peak, to that of NHase having αCys112-SO_2H and αCys114-SO_2H (named as $NHase_{(dSO2H)}$).[27,49] The relative abundance of the mass peak with *m*/*z* 1394 to the sum of the 1394 and 1407 peaks asymptotically increased with the increase in the amounts of Cpx and then

(48) Brennan, B. A.; Cummings, J. G.; Chase, D. B.; Turner, I. V., Jr.; Nelson, M. J. *Biochemistry* **1996**, *35*, 10068−10077.

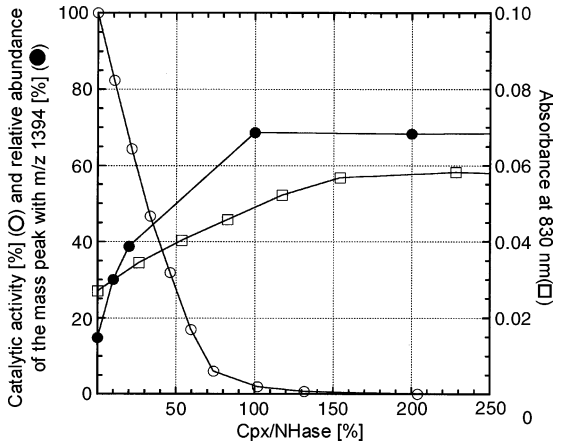

Figure 4. Effect of the addition of Cpx on the NHase activity (○), on the relative abundance of the mass peak with *m*/*z* 1394 (●) and on the absorbance at 830 nm of NHase (□). Native NHase (129 μM) was incubated with authentic Cpx at the indicated molar ratios (Cpx/NHase) for 30 min. NHase activity was measured with 20 mM methacrylonitrile as the substrate. The specific activity of native NHase was 1.2×10^3 units/mg protein. The relative abundance of the mass peak with *m*/*z* 1394 (R_{1394}) was calculated by the following equation: $R_{1394}\,(\%) = 100 \times H_{1394}/(H_{1394} + H_{1407})$, where H is the height of the mass peaks with *m*/*z* 1394 and 1407.

was saturated at about 70% over the molar ratio of 1:1 (NHase: Cpx) (Figure 4, closed circle). The relative abundance of the 1394 peak in NHase incubated with Cpx at a molar ratio of 1:1.2 (NHase:Cpx) reached up to 90%, when the dissociated α-subunit was removed by gel permeation chromatography (data not shown). No specific modification was detected in other tryptic digests of NHase incubated with Cpx at a molar ratio of 1:1 (NHase:Cpx). These results indicated that the decrease in the catalytic activity is ascribed to the oxidation of αCys114-SOH. The saturation at 70% may be attributed to the partial dissociation of the α- and β-subunits. Thus, we concluded that Cpx reacted stoichiometrically with the iron center to kill NHase irreversibly via the oxidation of αCys114-SOH to Cys-SO_2H.

UV−vis Absorption Spectral Changes Induced by Authentic Cpx. The UV−vis spectral changes induced by the addition of authentic Cpx (Figure 5A) were similar to those by commercially available *iso*-BN reagent (Figure 2). Namely, with an increase in the Cpx concentration, the peak at 711 nm with a shoulder at 395 nm diminished and a peak at 690 nm and shoulder peaks of about 340, 440, and 830 nm appeared. As shown in Figure 4 (open square), the absorbance changes at 830 nm almost saturated at the molar ratio of 1:1.5 (NHase: Cpx). This result suggested that 1 molar equiv of Cpx oxidized αCys114-SOH to Cys-SO_2H, and the rest was associated with $NHase_{(dSO2H)}$. To examine the interaction between Cpx and $NHase_{(dSO2H)}$, we measured the visible absorption spectra of $NHase_{(dSO2H)}$ in the absence and presence of Cpx (Figure 5B). $NHase_{(dSO2H)}$ exhibited a peak at 696 nm with shoulders at 370 and 440 nm in the absence of Cpx, whereas after the addition of Cpx at a molar ratio of 1:0.8 (NHase:Cpx), it showed a spectrum very similar to that of NHase incubated with 1.5 molar equiv of Cpx. The absorption spectrum did not change under aerobic conditions at least after 1 day. Thus, we concluded that Cpx formed a stable complex with $NHase_{(dSO2H)}$.

(49) The mass peaks at the *m*/*z* 1394 and 1407 were identified as the $[M + 2H]^{2+}$ ion of NK24 with carboxymethylated (SCM-) αCys109, αCys112-SO_2H, and αCys114-SO_2H and as that with SCM-αCys109, αCys112-SO_2H, and SCM-αCys114 by MS/MS sequencing, respectively.

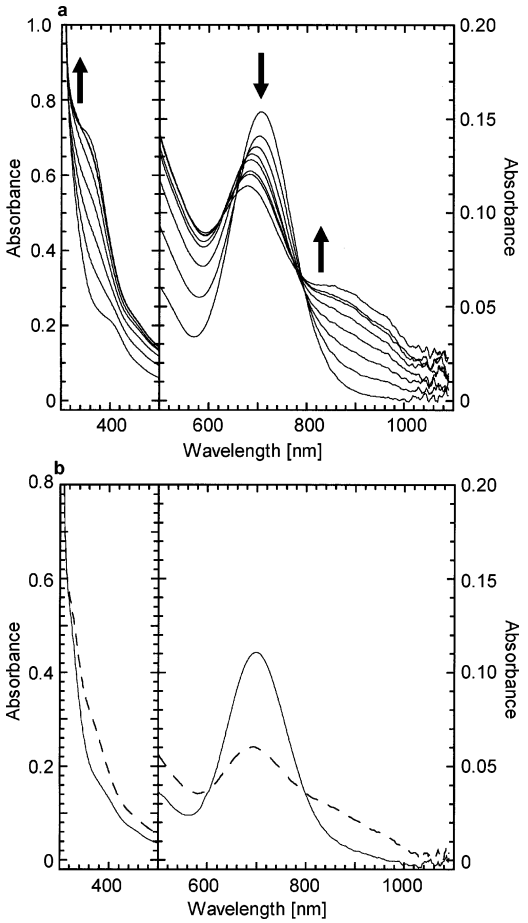

Figure 5. UV−vis absorption spectra of NHase (a) and $NHase_{(dSO2H)}$ (b) in the presence of Cpx. (a) Each spectrum was measured 30 min after the addition of authentic Cpx at molar ratios of NHase:Cpx = 1:0.0, 1:0.26, 1:0.54, 1:0.83, 1:1.2, 1:1.5, 1:2.3 and 1:6.4. NHase was dissolved in buffer A at a concentration of 129 μM. All spectra were recorded at room temperature with a V-570 UV/vis/NIR spectrophotometer (JASCO Co., Ltd, Japan). (b) The concentrations of Cpx were 0 μM (solid line) and 100 μM (dashed line), respectively. $NHase_{(dSO2H)}$ was dissolved in buffer B at a concentration of 130 μM. $NHase_{(dSO2H)}$ was prepared as described in the Experimental Section. All spectra were recorded at room temperature with a Carry50 UV/vis spectrophotometer (Varian Co., Ltd, USA).

Mass Spectrometric Analyses of the Oxidation of αCys114-SOH by Cpx. The mechanism for the oxidation of αCys114-SOH by Cpx was studied by MALDI-TOF MS in combination with the ^{18}O-labeling experiment. Figure 6A shows the MALDI-TOF mass spectrum of NK24 isolated from NHase that had been inactivated by Cpx in the buffer A containing normal H_2O (natural abundance, ^{16}O:^{17}O:^{18}O = 99.76:0.04:0.20). The mass spectrum showed five major isotopic peaks, and the *m*/*z* value of the monoisotope molecular species was 2786.0, corresponding to the $[M + H]^+$ ion of NK24 having one carboxymethylated cysteine (Cys-SCM) and two Cys-$S^{16}O^{16}OH$'s (Figure 6A). Meanwhile, the isotope pattern of NHase oxidized by Cpx in buffer containing ^{18}O-enriched water (81 atom %^{18}O) was more complicated (Figure 6B). We assumed that there were two kinds of molecular species whose monoisotopes had the *m*/*z* values 2786 and 2788. The latter value corresponds to the $[M + H]^+$ ion of NK24 having αCys109-SCM, αCys112-$S^{16}O^{16}OH$, and αCys114-$S^{16}O^{18}OH$. The incorporation of ^{18}O to the sulfur atom of αCys114, not to that of αCys112, was confirmed by MS/MS sequencing. To confirm this assumption, we calculated the theoretical distribution of isotopes by providing that the NK24

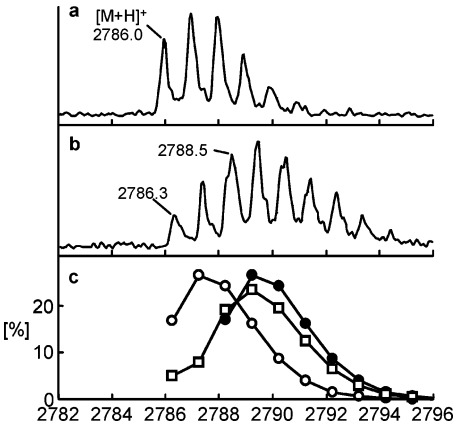

Figure 6. Oxidation of αCys114-SOH by Cpx in the presence of $H_2{}^{18}O$. MALDI-TOF mass spectra of NK-24 isolated from NHase incubated with Cpx at a molar ratio of NHase:Cpx = 1:2 in buffer A containing normal H_2O (natural abundance, ^{16}O:^{17}O:^{18}O = 99.76:0.04:0.20) (a) and containing ^{18}O-enriched H_2O (81 atom % ^{18}O) (b). The method for ^{18}O-labeling was described in the Experimental Section. (c) The theoretical molar distribution of stable isotopes in NK24 having αCys114-SO_2H. (○) 100% αCys114-$S^{16}O^{16}OH$; (●) 100% αCys114-$S^{16}O^{18}OH$; (□) a mixture of 70% αCys114-$S^{16}O^{18}OH$ and 30% αCys114-$S^{16}O^{16}OH$.

sample had 70% αCys114-$S^{16}O^{18}OH$ and 30% αCys114-$S^{16}O^{16}OH$ (Figure 6C). The calculated distribution was in good agreement with that of NHase treated in $H_2{}^{18}O$-enriched buffer (Figure 6B). These results strongly suggested that an oxygen atom of solvent water was incorporated into the SOH group of αCys114 during the oxidation of NHase by Cpx.

Discussion

Cpx was first synthesized in 1960 as a quite stable hydroperoxide under aerobic conditions.[50] Indeed, pure Cpx is stable above 120 °C. We confirmed the contamination by Cpx of the commercially available *iso*-BN reagents purchased from five different companies, although the amount of Cpx varied in each reagent. These results suggested that contamination by Cpx is widespread in commercially available *iso*-BN reagents. It should be noted that, after 6 months of storage at 4 °C, the distilled *iso*-BN induced visible absorbance changes in NHase similar to those observed on the addition of Cpx and inactivated the enzyme (data not shown). Therefore, we concluded that Cpx is at least partially generated from *iso*-BN itself in an autooxidative manner. Although the mechanism by which Cpx is generated in commercially available *iso*-BN is not known, it is possible that the isopropyl group in *iso*-BN suffers autooxidation in air to generate Cpx.

The oxidation of Cys-SOH as well as cysteine (Cys-SH) in proteins including NADH oxidase,[51] protein tyrosine phosphatase,[52] peroxiredoxin,[51,53] and alkyl hydroperoxide reductase[54] by hydroperoxide molecules has been well investigated. Also, it was reported that the thiolato ligands of some model compounds mimicking the structure of the NHase metal centers were oxidized to sulfenato or sulfinato structures by treatment with H_2O_2.[34,55−58] However, in these model compounds, it was not clear how H_2O_2 molecules oxidize the thiolato ligands. In the present study, we confirmed that, when αCys114-SOH was oxidized to Cys-SO_2H by Cpx, one oxygen atom was provided from the solvent H_2O by using MALDI-TOF MS combined with ^{18}O-labeling experiments (Figure 6).

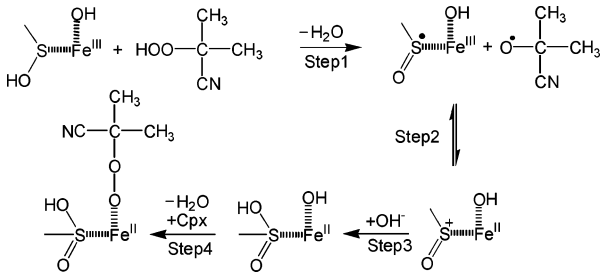

Figure 7. Possible reaction mechanism of Cpx with the catalytic iron center of NHase. Cpx oxidizes the sulfur atom of αCys114-SOH to produce NHase having αCys114-S•(=O)···Fe^{III} (step 1). αCys114-S•(=O)···Fe^{III} exists in equilibrium with αCys114-S^{+}•(=O)···Fe^{II} (step 2). OH^- from the solvent attacks the positively charged sulfur atom of αCys114-S^{+}•(=O)···Fe^{II}, to form Cys-SO_2H ($NHase_{(dSO2H)}$) (step 3). Another Cpx molecule binds directly to the iron(III) ion of $NHase_{(dSO2H)}$ to form a stable complex, 2-cyano-2-propyl-hydroperoxo-$NHase_{(dSO2H)}$ (step 4).

Cpx specifically modifies αCys114-SOH to Cys-SO_2H to inactivate NHase (Figure 4). Since no specific modification was detected in the other amino acid residues in α- and *β*-subunits, the sulfenic acid structure of αCys114 is indispensable for NHase to exhibit the catalytic activity. Very recently, we have found that NHase was inactivated by aerobic oxidation of αCys114-SOH to Cys-SO_2H.[59,44] Consistent with these results, Heinrich et al. reported that a bis(sulfenato-S) compound, Na[Co(L-N_2SOSO)(*t*BuNC)$_2$], catalyzed the hydration of acetonitrile, whereas its corresponding bis(sulfinato-S) complex Na[Co(L-$N_2SO_2SO_2$)(*t*BuNC)$_2$] was inactive.[34] Although the exact function remains unclear, the sulfenic acid group of αCys114 is likely to play a key role in the catalytic hydration reaction of NHase.

On the basis of the results obtained, we would like to propose a possible mechanism for the reaction of Cpx with the catalytic iron center of NHase (Figure 7). The characteristic spectral changes shown in Figure 5 are considered to originate from the interaction between the iron(III) atom of $NHase_{(dSO2H)}$ and Cpx. Cpx has two coordination groups, −CN and −OOH. Thus, we assume two binding forms: R−CN···Fe and R−OO···Fe. According to a previous paper, a model compound of Fe-type NHase $[Fe(III)(S_2{}^{Me2}N_3(Et,Pr))]^+$ exhibited spectral change by the association with acetonitrile.[60] The spectral change is similar to that observed for $NHase_{(dSO2H)}$ in the presence of Cpx, suggesting that the binding form is expressed as R−CN···Fe. However, we could not find any spectral changes of $NHase_{(dSO2H)}$ by addition of acetonitrile, propionitrile, acetone cyanohydrin,

(50) Talat-Erben, M.; Önol, N. *Can. J. Chem.* **1960**, *38*, 1154−1157
(51) Claiborne, A.; Yeh, J. I.; Mallet, T. C.; Luba, J.; Crane, E. J., III; Charrier, V.; Parsonage, D. *Biochemistry* **1999**, *38*, 15407−15416.
(52) Denu, J. M.; Tanner, K. G. *Biochemistry* **1998**, *37*, 5633−5642.
(53) Prouzet-Mauleon, V.; Monribot-Espagne, C.; Boucherie, H.; Lagniel, G.; Lopes, S.; Labarre, J.; Gari, J.; Lauquin, G. J. M. *J. Biol. Chem.* **2002**, *277*, 4823−4830.
(54) Yang, K. S.; Kang, S. W.; Woo, H., A.; Chae, H. Z.; Kim, K.; Rhee, S. G. *J. Biol. Chem.* **2002**, *277*, 38029−38036.
(55) Rat, M.; Alves de Sousa, R.; Vaissermann, J.; Mansuy, D.; Artaud, I. *J. Inorg. Biochem.* **2001**, *84*, 207−213.
(56) Taylor, L. A.; Noveron, J. C.; Olmsteas, M. M.; Mascharak, P. K. *Inorg. Chem.* **2000**, *39*, 357−362.
(57) Taylor, L. A.; Noveron, J. C.; Olmsteas, M. M.; Mascharak, P. K. *Inorg. Chem.* **1999**, *37*, 1138−1139.
(58) Heinrich, L.; Li, Y.; Vaissermann, J.; Chottard, G.; Chottard, J. C. *Angew. Chem., Int. Ed.* **1999**, *38*, 3526−3528.
(59) *n*-Butyric acid was shown to be a competitive inhibitor for Fe-type NHase of *Rhodococcus* sp. R312 with a K_i value of 0.9 mM (Kopf, M.; Bonnet, D.; Artaud, I.; Pétré, D.; Mansuy, D. *Eur. J. Biochem.* **1996**, *240*, 239−244) and has been widely used as a stabilizing agent for Fe- and Co-type NHases.
(60) Shearer, J.; Jackson, H. L.; Schweitzer, D.; Rittenberg, D. K.; Leavy, T. M.; Kaminsky, W.; Scarrow, R. C.; Kobacs, J. A. *J. Am. Chem. Soc.* **2002**, *124*, 11417−11428.

and pure isobutyronitrile. From these results, the possible binding form is assumed to be formulated as R−OO···Fe (Figure 7, Step 4). Further, the binding of the hydroperoxo group of Cpx to the iron(III) ion of $NHase_{(dSO2H)}$ was strongly suggested because similar absorption spectral changes were observed in the NHase treated with H_2O_2 or *tert*-butyl hydroperoxide (data not shown).

Reactions between non-heme iron complexes and hydroperoxide compounds have been well investigated.[61−64] In general, H(or R)−OO···Fe is not so stable and undergoes homolytic or heterolytic cleavages. The $NHase_{(dSO2H)}$−Cpx complex is very unique because of its stability even under aerobic condition. The sulfinato ligands might be significantly important in the stabilization of the complex.

Conclusions

We found commercially available *iso*-BN to contain 2-cyano-2-propyl hydroperoxide (Cpx). Authentic *iso*-BN was not a strong competitive inhibitor reported previously[35,36] and hydrolyzed by the Fe-type NHase from *Rhodococcus* sp. N771 with the kinetic parameters $K_m = 4.7$ mM and $V_{max} = 1.0 \times 10^3$ units/mg protein. It is Cpx that inhibited the activity of NHase in commercially available *iso*-BN. The present study well explains why the hydration activity of Fe-type NHase for *iso*-BN varied in previous papers.[35−41] Cpx functions as a novel inhibitor for NHase: it irreversibly inactivates NHase via specific oxidation of αCys114-SOH to Cys-SO_2H ($NHase_{(dSO2H)}$) by a 1:1 molar reaction. Although further investigation is required for understanding the reaction between Cpx and NHase in detail, the present study demonstrated that the sulfenic acid group of αCys114 plays a key role in the catalytic hydration reaction of NHase. Cpx may give a clue for understanding the catalytic mechanism of NHase. Also, Cpx may be a useful reagent for the specific oxidation of Cys-SOH of NHase.

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(61) Mathé, C.; Mattioli, T. A.; Horner, O.; Lombard, M.; Latour, J.-M.; Fontecavem M.; Niviére, V. *J. Am. Chem. Soc.* **2001**, *124*, 4966−4967.
(62) Solomon, E. I.; Brunold, T. C.; Davis, M. I.; Kemsley, J. N.; Lee, S.-K.; Lehnet, N.; Neese, F.; Skulan, A. J.; Yang, Y.-S.; Zhou, J. *Chem. Rev.* **2000**, *100*, 235−349.
(63) Lehnet, N.; Neese, F.; Ho, R. Y. N.; Que, L., Jr.; Solomon, E. I. *J. Am. Chem. Soc.* **2002**, *124*, 10810−10822.
(64) Hazell, A.; McKenzie, C. J.; Nielsen, L. P.; Schinder, S.; Weitzer, M. *J. Chem. Soc., Dalton Trans.* **2002**, 310−317.