Highlight Review

Structural and Functional Model Systems for Analysis of the Active Center of Nitrile Hydratase

Takuma Yano, Tomohiro Ozawa, and Hideki Masuda*

Abstract

The unique active site structure of nitrile hydratase (NHase) has a central metal ion (Co^{III} or Fe^{III}) coordinated by two amide nitrogens from the peptide backbone, one cysteine sulfur and two oxidized cysteine sulfurs (Cys–SO and Cys–SO_2). In this review, the biological implications of the nitrile hydration mechanism are discussed in context of model complexes prepared with the aim of understanding the unique structure of nitrile hydratase.

Figure 1. Active site structure of Co-type NHase from *Pseudonocardia thermophilia JCM 3095*.

♦ Introduction

The well-known natural enzyme-catalyzed reactions of nitrilase and nitrile hydratase (NHase) are responsible for metabolism of highly toxic nitrile compounds: Nitrilase hydrolyzes nitrile compounds to their corresponding carboxylic acids and ammonia in one step.[1] NHase hydrates a nitrile to provide the corresponding amide product via an addition reaction of one water molecule. This reaction is followed by complete decomposition to the corresponding carboxylic acid and ammonia in an amidase-catalyzed reaction. The nitrile hydration reaction is a very important industrial process for producing products such as acrylamide.[2] The use of NHase in industrial processes is advantageous, because it produces fewer by-products and is relatively stable. NHase is a metalloenzyme with an active site octahedral Fe^{III} or Co^{III} ion. Structural analyses by X-ray crystallography for both types of NHases have revealed that they have the same coordination geometry at the active center.[3] The central metal ion is equatorially coordinated by two amide nitrogens from the peptide backbone and two oxidized cysteine sulfurs (Cys–SO and Cys–SO_2) and axially coordinated by one cysteine sulfur and either NO (in Fe-type NHases)[4] or H_2O (in Co-type NHases)[5] (Figure 1). These coordination environments have three characteristics that distinguish the active centers of NHases from the active centers of other metalloenzymes: 1) They have three cysteine ligands in different oxidation states including sulfur (S), sulfenyl (SO), and sulfinyl (SO_2) groups; 2) Two amidate nitrogens from the peptide backbone are coordinated; and 3) The ligands in the equatorial plane are in an asymmetric arrangement with (5–5–6)-membered chelate rings. These unique structural features make it difficult to understand the relationships between the coordination environment and the hydration mechanism. Three reaction processes have been proposed (Figure 2):[6] (i) A coordinated hydroxide with nucleophilicity provided by the water binding precursor in the active site directly attacks a coordinated nitrile. (ii) The hydroxide species generated accord-
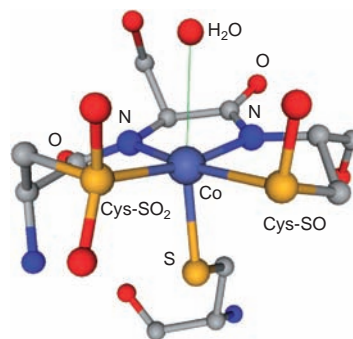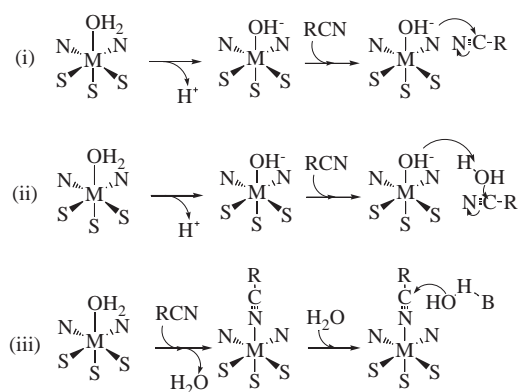

Figure 2. Proposed reaction mechanisms for NHase.

ing to mechanism (i) activates a surrounding water molecule, and the activated water molecule attacks a substrate. (iii) Nitrile substrate activated by coordinating to the metal ion is attacked by a water molecule via an outersphere reaction. In order to understand the reaction mechanism, many chemists have synthesized and characterized model metal complexes and proposed roles for the unique coordination environment in promoting the hydration reaction. In this review, we will discuss the relationships between the enzymatic activity and the unique structure of the NHase active site on the basis of the results obtained by investigations of model compounds.

♦ Function of the Post-translationally Oxidized S Atoms

Coordination of oxidized cysteine residues (sulfenate and sulfinate groups) in the active site of NHase is quite unique. A

*Dr. Takuma Yano, Dr. Tomohiro Ozawa, Prof. Hideki Masuda**
Graduate School of Engineering, Nagoya Institute of Technology, Gokiso-cho, Showa-ku, Nagoya 466-8555
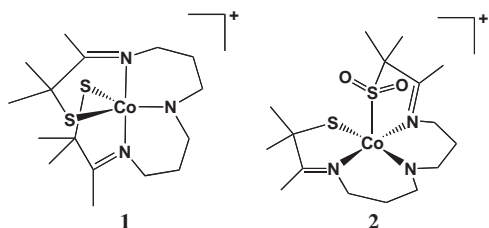*E-mail: masuda.hideki@nitech.ac.jp*

Figure 3. Structures of anion parts of **1** and **2**. $[Co^{III}S_2{}^{Me2}N_3(Pr,Pr)]^+$ (**1**) and $[Co^{III}S^{Me2}(S^{O_2})N_3(Pr,Pr)]^+$ (**2**).

Figure 4. Structures of the $[Fe^{III}(PyPepS)_2]^-$ anion part of **3**, the $[Fe^{III}(PyPepSO_2)_2]^-$ anion part of **4**, and the $[Fe^{III}(PyPepSMe)_2]^+$ cation part of **5**.

Figure 5. Structures of the $[Co(PyPS)(CN)]^{2-}$ anion part of **6** and the $[Co(PyS(SO_2)(CN))]^{2-}$ anion part of **7**.

recent study[7] demonstrated that atmospheric oxygen promotes the oxygenation of sulfur atoms in reconstituted NHase to advance the enzymatic reaction. Since hydration of nitriles is not observed in the non-S-modified reconstituted enzyme, it was concluded that the post-translational modification at the active site of NHase is one of the essential factors for the catalytic reaction.[7] In addition, further oxidation of the sulfenate to sulfinate caused an inactivation of the enzymatic reaction.[8] This also suggests that the sulfenate group is an important factor for maintaining the activity and is expected to play a different role than the sulfinate group. Many studies using model complexes have focused on the effect of the oxygenated sulfurs. Several Co^{III} and Fe^{III} complexes with tridentate, tetradentate, or pentadentate ligands, which contain sulfur atoms and an imine or an amide group, have been synthesized and characterized.[9–19]

Kovacs and co-workers have prepared a Co^{III} complex with an amine-type ligand, $[Co^{III}S_2{}^{Me2}N_3(Pr,Pr)]PF_6$ (**1**) (Figure 3).[9] The azide and thiocyanate ligands were found to bind to the central metal ion in the complex **1** at a position trans to one of the thiolates at room temperature. In kinetic studies, the dissociation constants (K_d) of azide and thiocyanate from **1** were estimated to be $(2.14 \pm 0.50) \times 10^{-2}$ and $(7.22 \pm 0.04) \times 10^{-1} s^{-1}$, respectively. These rates are comparable to those of the corresponding Fe^{III} complexes, although complex **1** is a kinetically inert Co^{III} complex with a low-spin six-coordinate geometry. A penta-coordinate square-pyramidal complex, $[Co^{III}S^{Me2}(SO_2)N_3(Pr,Pr)]PF_6$ (**2**), was also prepared, in which one of the thiolates of **1** was oxidized to sulfinate (Figure 3).[9] In contrast to **1**, neither azide nor thiocyanate was found to coordinate to the vacant site of **2**. This was attributed to the strong trans effect induced by the sulfinate group. It was concluded that the ligand-exchange reaction in the low-spin Co^{III} complex was likely promoted by positioning of the ligand trans to the thiolate sulfur atom.

Harrop and Mascharak focused on the roles of functional groups of two amide nitrogens and three sulfurs with different oxidation states in the active center of NHase. Fe^{III} complexes with amide-type tridentate ligands, $[Fe^{III}(PyPepS)_2]^-$ (**3**), $[Fe^{III}(PyPepSO_2)_2]^-$ (**4**), and $[Fe^{III}(PyPepSMe)_2]^-$ (**5**) (Figure 4) were synthesized and characterized.[10] The effect of oxygenation of the coordinated sulfur atoms was investigated by cyclic voltammetry measurements for **3**, **4**, and **5**, because the redox potentials of the central metal ion are necessarily reflected by the different oxidation states. The redox half potential ($E_{1/2}$) of **3** corresponding to Fe^{III}/Fe^{II} is -1.12 V vs. SCE in DMF, which is sufficient to maintain the $3+$ oxidation state of the Fe center. The redox potential of complex **5**, whose sulfur atoms are neutral thioethers, is 0.27 V vs. SCE, a much higher potential than that of **3** which has thiolate (S) donors. This is be-
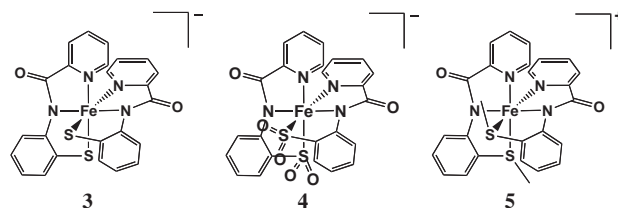
cause the electron-donating character is weakened by the neutralization of the thiols. Complex **4** exhibits a quasi-reversible redox behavior at -0.32 V vs. SCE. The positive shift in the potential shows a similar tendency as that of **5**, implying that oxygenation of the coordinated sulfur atoms to sulfinates decreases the electron-donating character of the S atom as a result of the presence of the electron-withdrawing oxygen atoms.
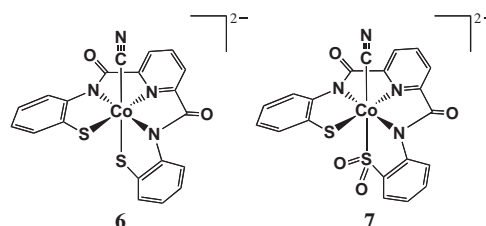
A Co^{III} complex with a pentadentate amide-type ligand, $[Co(PyPS)(CN)]^{2-}$ (**6**) (Figure 5) was also characterized.[11] The coordinated monodentate cyanide in complex **6** was easily replaced by a water molecule to form $[Co(PyPS)(H_2O)]^-$. The acid dissociation constant (pK_a) of the coordinated water molecule in $[Co(PyPS)(H_2O)]^-$ was determined to be 8.3 ± 0.03. Interestingly, the water molecule was found to be capable of catalyzing hydration of acetonitrile to acetamide in aqueous buffer solution at pH 9.5 at 50 °C and the TON was found to be 15 after 2 h and 18 after 4 h.[11] Oxygenation of one sulfur atom to sulfinate in **6** gave complex **7** (Figure 5).[12] The coordinated CN^- ion was replaced by a water molecule at a much slower rate than that of the same reaction by **6**. The replaced H_2O species showed evidence of proton dissociation with pK_a 7.2 ± 0.06, which is lower than that of **6** and similar to physiological pH. Therefore, the rate of hydration of acetonitrile catalyzed by **7** is 3 times faster than that of **6** at pH 8.0.[12] These results indicate that the sulfur oxygenation increases the Lewis acidity of the metal center. The post-translational oxidation of sulfur in NHase was described as playing an important role in the hydration of the substrate, because the decrease of the pK_a in the coordinated water molecule to physiological pH facilitates deprotonation of the water molecule (Figure 2(i) or 2(ii)).

Some metal complexes with N_2S_2-type ligands have been synthesized and characterized from a viewpoint of their unique coordinating ligands in the equatorial plane of the NHase active site. Co^{III} complexes with two amide nitrogens donor atoms, $[Co(N_2S_2)(tBuNC)_2]^-$ (**8**) and $[Co(N_2(SO_2)_2(tBuNC)_2)]^-$ (**9**), were prepared by Artaud and co-workers (Figure 6).[13] Complex **8** has ^1H NMR signals in the diamagnetic region at 250 K,
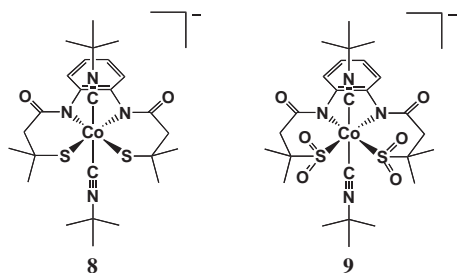
Figure 6. Structures of the [Co(N$_2$S$_2$)(t-BuNC)$_2$]$^-$ anion part of **8** and the [Co{N$_2$(SO$_2$)$_2$}(t-BuNC)$_2$]$^-$ anion part of **9**.
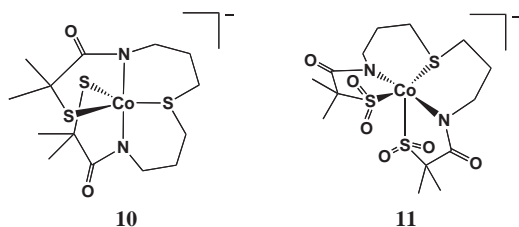
Figure 7. Structures of the [Co(L:N$_2$S$_3$)]$^-$ anion part of **10** and the [Co(L:N$_2$S$_3$–O$_4$)]$^-$ anion part of **11**.

indicating that it has octahedral geometry in solution. An increase in temperature to 300 K gives rise to signals in the paramagnetic region. These signals were attributed to a [Co(N$_2$S$_2$)]$^-$ species produced by dissociation of the axial ligands ([Co-(N$_2$S$_2$)]$^-$:**8** = 37:63). This result indicates that the coordination affinity of the axial t-BuNC ligand of **8** is smaller than that of **9**.

Yano et al. also synthesized two new Co^{III} complexes, [Co(L:N$_2$S$_3$)]$^-$ (**10**) and [Co^{III}(L:N$_2$S$_3$–O$_4$)]$^-$ (**11**), which contain two amide nitrogens, two thiolates, or sulfinates, and a thioether as donor atoms (Figure 7).[14] Spectrophotometric coordination equilibrium experiments with t-BuNC for **10** and **11** were carried out in EtOH. The constants (log K) were found to be 2.70 for **10** and 4.57 for **11**. The constant for **11** is two orders larger in magnitude than that of **10**. It was also demonstrated that H$_2$O or EtCN could coordinate to the vacant site of complex **11** as well as t-BuNC but did not react with **10**.[14b] These results indicate that the Lewis acidity of central metal ion of **11** is greater than that of **10**.

Three N$_2$S$_2$-type Co^{III} complexes, [Co^{III}(L$_{C=O}$:N$_2$S$_2$)-(t-BuNC)$_2$]$^-$ (**12**), [Co^{III}(L$_{C=O}$:N$_2$(SO)$_2$)(t-BuNC)$_2$]$^-$ (**13**), and [Co^{III}(L$_{C=O}$:N$_2$(SO$_2$)$_2$)(tBuNC)$_2$)]$^-$ (**14**), were also synthesized and characterized. The sulfur atoms were found to exist in different oxidation states (Figure 8).[15] Electronic absorption (UV–vis), infrared spectral (IR), and TG measurements demonstrated that the bond strengths between the Co^{III} ion and the C atoms of the coordinated t-BuNC have the following trend: **12** < **13** < **14**. DFT calculations were performed for **12**, **13**, and **14**, and the charge densities of all of the atoms were estimated by Mulliken population analysis. The calculated atomic charges on the Co^{III} centers were +1.466, +1.536, and +1.542 for **12**, **13**, and **14**, respectively. The observation of an increase in Lewis acidity of the metal ion upon oxygenation of the sulfur atoms provides support for the experimental results described above.

Thus, the results described above demonstrate that oxygenation of the sulfur ligands has the effect of increasing the Lewis
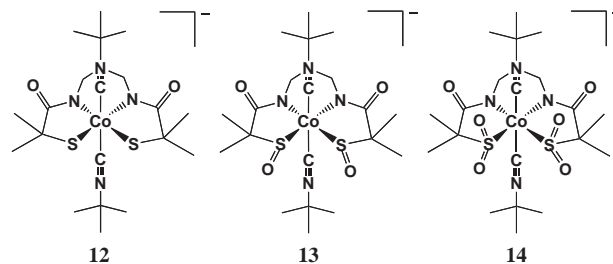

Figure 8. Structures of the [Co(L$_{C=O}$:N$_2$S$_2$)(t-BuNC)$_2$]$^-$ anion part of **12**, the [Co(L$_{C=O}$:N$_2$(SO)$_2$)(tBuNC)$_2$]$^-$ anion part of **13**, and the [Co(L$_{C=O}$:N$_2$(SO$_2$)$_2$)(tBuNC)$_2$]$^{2-}$ anion part of **14**.

acidity of the metal center, making it capable of binding and activating a water molecule or a substrate molecule. In NHase, different oxygenation states were identified for three sulfur ligands. It has been clarified that the sulfinate and sulfenate groups are both required for catalysis of the hydration reaction,[8] although the sulfenate is a generally unstable functional group and is considered to be less effective than the sulfinate group on increasing the Lewis acidity, as suggested by the theoretical investigations described above.[15] In the crystal structures of NHases, the oxygenated sulfurs appear to interact with two arginine residues. This has significant implications for the enzymatic activity. In order to understand this behavior, IR spectral measurements were performed for **13** and **14** in solution. Complex **13** exhibits a clear solvent-dependent S–O stretching frequency. In contrast, such solvent-dependence was not observed for the sulfinate groups of **14**, indicating that the sulfenyl oxygen atom has nucleophilic character. Other investigations suggest that the sulfenyl oxygen is nucleophilic. Na[Co^{III}(L-N$_2$SOSO)(tBuNC)$_2$] (**15**) and (Me$_4$N)[Co^{III}(L-N$_2$SO$_2$SO$_2$)-(tBuNC)$_2$] (**16**) have been prepared by Chottard and co-workers (Figure 9).[16] Complex **15** hydrolyzes acetonitrile to acetoamide but **16** does not. It was proposed that the sulfenate oxygen in **15** is nucleophilic and attacks the nitrile molecule. Kovacs et al. also reported that the sulfenyl group in [Fe^{III}(ADIT)-(ADIT–O)]$^+$ interacts electrostatically with a proton (**17**: Figure 9)[17] in solution at low temperature. Theoretical calculations were performed by Himo et al. on the first-shell mechanism for nitrile hydration in Fe-type NHase.[18] The calculated result indicates that the sulfenyl oxygen of Cys114–SO$^-$ might function as a catalytic base to abstract a proton from the attacking water molecule. Indeed, Noguchi et al. suggested that both the sulfinate and the sulfenate groups are in the deprotonated states with hydrogen-bonding interaction.[19] These findings on the basis of the NHase and the model studies suggest that the sulfenate
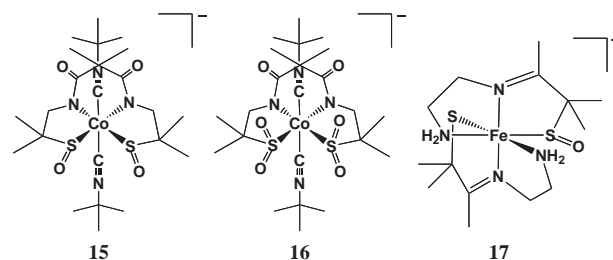

Figure 9. Structure of the [Co^{III}(L-N$_2$SOSO)(t-BuNC)$_2$]$^-$ anion part of **15**, the [Co^{III}(L-N$_2$SO$_2$SO$_2$)(t-BuNC)$_2$]$^-$ anion part of **16**, and the [Fe^{III}(ADIT)(ADIT–O)]$^+$ anion part of **17**.

oxygen in native NHase acts as a base (proton acceptor) and contributes to the activation of a water molecule and/or nitrile molecule.

Thus, the increase in the Lewis acidity of the metal center caused by the oxygenated sulfur ligands is one of the significant factors in the reactions with water molecules or nitrile subsrates. It was demonstrated that the sulfenate group interacts electrostatically with external molecules or cations. This suggests that the sulfenyl group acts as a base.

◆ *Function of the Amidate Nitrogen Donors from Peptide Backbone*

Coordination of an amidato group is another characteristic feature of the active sites of NHases. Examples of amidato coordination in the active sites have been reported for certain metalloenzymes.[20] Researchers have reported on the effect of amidato coordination to the metal center using nonoxygenated sulfur ligands. In this section, the influence of N^- coordination on the metal center in model complexes is described from a biological viewpoint.

An Fe^{III} complex **18** (Figure 10) with imine nitrogens as donor atoms was prepared by Mascharak with the aim of comparing complex **3** (Figure 4) with amide complexes.[21] In electrochemical studies carried out by cyclic voltammetry, complexes **3** and **18** were found to exhibit redox waves ($E_{1/2}$) at -1.12 and -0.13 V vs. SCE, respectively, which are assignable to the Fe^{III}/Fe^{II} couple. The large positive shift of the potential indicates that a strong ligand field provided by amidato coordination stabilizes the Fe^{3+} oxidation state sufficiently to promote hydration activity.[21]

Artaud and co-workers prepared the Co^{III} complex with an amide-type tetradentate ligand which provides coordination of ligands in a manner similar to that of the ligands in the equatorial plane of the NHase active site, $[Co(N_2S_2)]^-$ (**19**) (Figure 10).[22] The half potential ($E_{1/2}$) of **19** corresponding to the Co^{III}/Co^{II} redox behavior was -1.13 V vs. SCE in DMF. The potential is also low enough to stabilize the Co^{3+} oxidation state. The coordination behavior of monodentate ligands bound to **19** was investigated by means of UV–vis, IR, and ^1H NMR spectroscopic methods. Two CN^- ions or two NO molecules coordinate to the vacant axial sites of **19**, while imidazole, H_2O, and HO^- do not. This indicates that **19** does not have sufficient Lewis acidity to bind a ligand with σ- and/or π-donating character.[22] Thus, too much electron donation by a strong ligand field is unfavorable for the donation of monodentate ligands, such as OH^-, that are candidates for the reaction intermediates shown in Figure 2(i) or 2(ii).

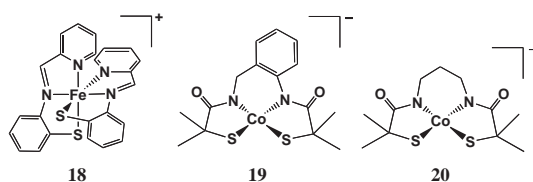Oxygenation of the sulfur atoms may compensate for the strong donation of coordinated amidato ligands, as described in the previous section. It would be instructive to understand the benefits of amidato coordination for NHases. In the crystal structure of NHase, the amidate oxygens electrostatically interact with water molecules that form a water channel connected to the oxygen atom of βTyr68. A recent study of the βY68F mutant of Co-type NHase demonstrated that the mutation causes a drastic decrease in the enzymatic activity.[23] In attempts to mimic the electrostatic interactions of this mutant, a square planar Co^{III} complex with an amide-type ligand, $[Co(L_{C=O}:N_2S_2)]^-$ (**20**), was prepared.[24] CN^- as well as t-BuNC,[16] NO,[24b] and $EtCN$[24c] were found to coordinate to the vacant sites of **20** and to other Co^{III} model complexes.[13,14,17,22] Solvent dependence on the coordination behavior was investigated in order to estimate influence of the carbonyl oxygens on the outer sphere. Absorption spectral changes were measured to determine the coordination equilibrium constant (K) for binding of t-BuNC to **20** in various solvents.

$$[Co(L_{C=O}:N_2S_2)]^- + 2t\text{-BuNC} \overset{K}{\rightleftharpoons} [Co(L_{C=O}:N_2S_2)(t\text{-BuNC})_2]^-$$
(1)
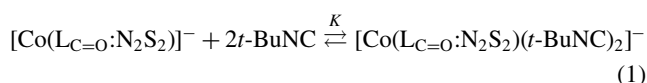
The log K values linearly depend upon the acceptor numbers (AN) of solvents, wherein AN is an index measure of electrophilicity of the solvent.[25] This result indicates that an electrophilic interaction of a solvent molecule with **20** results in axial coordination of the solvent molecule. In order to confirm that the carbonyl oxygens interact with solvent molecules, IR spectra of **20** were measured in various solvents. The stretching vibration energy of C=O was found to linearly decrease as the electrophilic interaction of the solvent increases. Changes in the strength in the amidato coordination are also expected to be mediated through this interaction. Cyclic voltammetry measurements indicate quasi-reversible redox behavior assignable to the Co^{III}/Co^{II} couple. The half-wave potentials also linearly shift to the positive potential range in a manner dependent upon the acceptor number. These linear relationships indicate that interactions of carbonyl oxygens with solvent molecules cause an increase in Lewis acidity of the Co^{III} center to promote the coordination of a monodentate ligand at the axial site. On the basis of the linear relationship, the redox potentials in aqueous solution (AN: 54.8) and a non-interacting state (AN: 0) were evaluated to be -930 and -1890 mV, respectively. Thus, the interaction with water molecules is expected to produce approximately a 1 V positive shift relative to non-interacting species. Such a solvent effect was also observed in the case of complex **11**.

In the crystal structures of NHases, a hydrogen-bonding network with other water molecules that extends from βTyr68–OH of Co^{III}-type NHase is formed, as described above. On the basis of the electrophilicity of C=O moieties, the interaction of the coordinated amide carbonyl oxygen with water molecules in the outer sphere and tyrosine–OH may control the Lewis acidity of the Co^{III} ion.[24]

◆ *Effect of Asymmetrically Arranged Equatorial (6–5–5) Chelate Rings in the Active Site*

The specific coordination environment in the equatorial plane is arranged with asymmetric (6–5–5) membered chelate rings, as shown in Figure 1.[5] In this section, influences of the
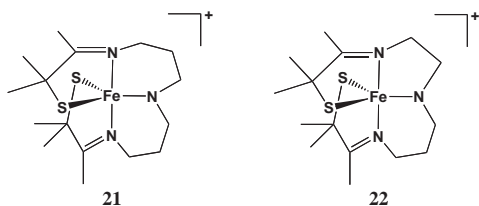

Figure 10. Structures of the $[Fe^{III}(PyAS)]^+$ cation part of **18**, the $[Co(N_2S_2)]^-$ anion part of **19**, and the $[Co(L_{C=O}:N_2S_2)]^-$ anion part of **20**.

Figure 11. Structures of $[Fe^{III}(S_2{}^{Me2}N_3(Pr,Pr))]^+$ (**21**) and $[Fe^{III}(S_2{}^{Me2}N_3(Et,Pr))]^+$ (**22**).

size and the arrangement of the chelate rings on the character of metal ion are discussed.

Focusing on the size of chelate rings, Kovacs and co-workers have prepared Fe^{III} complexes with amine-type ligands, $[Fe^{III}S_2{}^{Me2}N_3(Pr,Pr)]PF_6$ (**21**) and $[Fe^{III}S_2{}^{Me2}N_3(Et,Pr)]PF_6$(**22**) (Figure 11).[26] The former complex has a (5–6–6–5) arrangement while the latter has a (5–5–6–5) state. On the basis of the crystal structures, the τ value, which provides an index of the structural distortion from a square pyramid ($\tau = 0$) or a trigonal bipyramid ($\tau = 1$), was estimated to be 0.763 for **21** and 0.539 for **22**, indicating that the extra methylene group of **22** causes a trigonal distortion in the structure of the complex. Consequently, the space constructed by the S–Fe–N(amine) angle was opened by approximately $10°$ by the distortion. An azide molecule coordinates to a position trans to the thiolate in both complexes **21** and **22**. The coordination equilibrium was studied and evaluated. The dissociation equilibrium constant of **21** was found to be approximately 10 times larger than that of **22**, because of the distortion.[26a] Nitriles including acetonitrile, propionitrile, *t*-butylnitrile, and benzonitrile were also found to coordinate to **22** at low temperature but did not coordinate to **21**.[26b] It was concluded that the small distortions induced by ligand constraint have a dramatic influence on the reactivity of the metal complex.

Investigations of $N(amide)_2S_2$-type model complexes have resulted in reproduction of the equatorial coordination feature found in the active site of the NHases, in which two amide nitrogens and two sulfurs are in the cis position relative to each other, but the arrangement of chelate rings in the model complexes were found to be symmetric (6–5–6) or (5–6–5) arrangement. In order to understand the role of asymmetric arrangements in NHase, a Co^{III} complex with an asymmetric chelate ring arrangement, $[Co^{III}(L_{asym}:N_2S_2)]^-$ (**23**), was prepared by Yano et al. using an $N(amide)_2S_2$-type ligand (Figure 12).[24c] Cyclic voltammetry was used to evaluate the influence of the asymmetric chelate environment on the metal center. The complex **20** which has a symmetric (5–6–5) arrangement exhibits a quasi-reversible redox wave with a potential of $-1.1\,V$ vs. Fc/Fc^+ in methanol. This value is lower than that of **23** ($-0.9\,V$) which has an asymmetric arrangement. This result suggests that **23** receives a ligand field perturbation which is weaker than that of **20**,
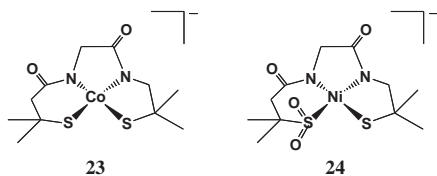
although the coordinating ligands and positions thereof are the same. Such a difference must be reflected in the Lewis acidity. Coordination behavior of *t*-BuNC to **23** was also investigated in order to compare it with that of **20**. The equilibrium constant ($\log K$) was calculated to be 4.40, which is approximately 10 times larger than that of **20** (3.13) in CH_2Cl_2. This indicates that **23** has a greater Lewis acidity than that of **21**.[24c] Indeed, this difference was observed in the coordination behavior of EtCN, one of the substrates of NHase. In EtCN solution, the electronic absorption spectrum of **23** at $-55°C$ is converted to a six-coordinate structure. On the other hand, approximately 70% of the four-coordinate species remains in **20** at the same temperature, as estimated from the spectral features. DFT calculations were performed on **20** and **23**. Mulliken population analysis indicated that the net atomic charges of the Co^{III} centers of **20** and **23** were $+1.054$ and $+1.068$, respectively. This result indicates that **23** has higher Lewis acidity than **20**, which is consistent with the experimental results described above.

With regard to the coordinating atoms of **23**, the electron density of the S atom in the six-membered chelate ring was interestingly much larger than that of the S atom in the five-membered chelate ring. This result implies that the S atom in the six-membered chelate ring is more likely to be oxidized than the S atom in the five-membered chelate ring. A Ni^{II} complex with the asymmetric $N(amide)_2S_2$-type ligand was isolated as a single crystal. The crystal structure demonstrated that the coordinated sulfur atom which resides in a six-membered chelate ring was oxidized by air to sulfinate, whereas another thiolate sulfur atom remains unoxidized ($[Ni^{II}(L_{asym}:N_2S(SO_2))]^{2-}$ (**24**): Figure 12).[27]

Thus, the asymmetric arrangement of the chelate ring in NHase may be important to retain asymmetric oxygenated sulfur states that are essential for hydration activity as well as for increasing the Lewis acidity of the metal center.

♦ *Hydrolysis Reaction of tBuNC by Using N_2S_3-type Model Complexes*

The use of NHase as a catalyst for the large scale preparation of acrylamide is a good example of a use of a metalloenzyme in an industrial processes. In biological studies, Odaka and co-workers have recently reported that *t*-BuNC also acts as a substrate for Fe-type NHase. In this system, it was found that this substrate was hydrolyzed to *t*-BuNH$_2$.[28]

Cyanide ions of complexes **6** and **7** can be replaced by water molecules in an aqueous solution as mentioned above. Increase of pH of the solution caused significant absorption spectral changes for both complexes. Coordination of water molecule to **11** in an aqueous solution was also observed,[14a] but they did not show any pH dependence in the range of 5.5–10.2,[29] indicating that the coordinated water was not deprotonated, which is in contrast with the complexes **6** and **7**. This result indicates that the bound water molecule is activated in **6** and **7** (the pK_a values are 8.3 and 7.2, respectively),[12] but not in **11**. On the other hand, EtCN and *t*-BuNC reversibly coordinate to the Co^{III} ion in **11**.[13] Hydrolysis of the *t*-BuNC was performed by **10**, **11**, and **25** (Figure 13).[29] The complex **11** with *t*-BuNC (**26**: Figure 13), and the complex **25** with *t*-BuNC, which includes sulfenate and/or sulfinate groups, catalytically hydrolyze the *t*-BuNC molecule to *t*-BuNH$_2$ and CO in aqueous buffer solution at pH 10.2 at
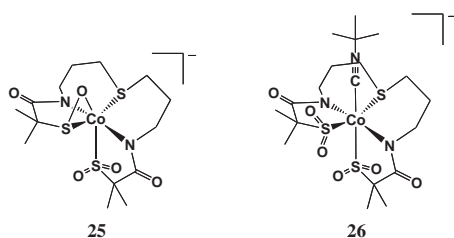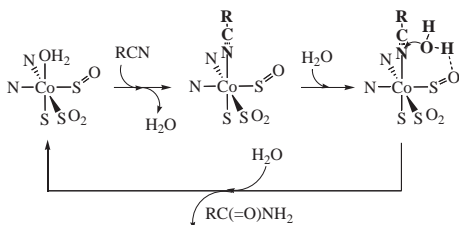

Figure 12. Structures of $[Co^{III}(L_{asym}:N_2S_2)]^-$ (**23**) and $[Ni^{II}(L_{asym}:N_2S(SO_2))]^{2-}$ (**24**).

Figure 13. Structure of $[Co(L:N_2S_3-O_3)]^-$ (**25**) and $[Co(N_2S_3-O_4)(t\text{-BuNC})]^-$ (**26**).

Scheme 1. Proposed hydration mechanism of nitrile in NHase.

40 °C (TON is 9.7 and 10.6, respectively),[29] while the complex **10** with *t*-BuNC does not. Sugiura et al. have reported nitrile coordination on the basis of the electronic absorption and ESR spectral measurements.[30] In light of these results, we propose that substrate coordination to the metal center is the probable pathway in the catalytic reaction.

♦ *Summary and Outlook*

In this review, the relationships between the enzymatic activity and the unique structure of the NHase active site have been discussed on the basis of data obtained from model compounds. The active center structure of NHase which has two amidate nitrogens and three sulfur atoms with different oxidation states is quite unique. These important studies have enabled us to propose the following functions for the roles of the unique structures of the active center of NHase. (i) The oxygenation of sulfurs contributes to an increase in the Lewis acidity of the metal center; (ii) the sulfenate group also acts as a base; (iii) the amidate coordination fine-tunes the Lewis acidity for binding of substrates via electrostatic interactions with the second coordination sphere; (iv) the asymmetric (6–5–5) chelate ring contributes to the selective oxygenation of the sulfur atom of the six-membered ring; and (v) hydrolysis of *t*-BuNC catalyzed by **11** and **25** indicates that the reaction is achieved through binding of the substrate to the complex. In Fe-type NHase, it has been reported that the substrate EtCN is hydrolyzed through coordination to the vacant axial site. On the basis of these results, we propose that the hydration/hydrolysis reaction of nitrile and isocyanide compounds proceeds via coordination of the substrates to the metal ion, followed by attack of a water molecule that has been activated by the sulfenyl group (Scheme 1).

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