# Two-Metal Ion, Ni(II) and Cu(II), Binding α-Helical Coiled Coil Peptide

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**Abstract:** Metalloproteins are an attractive target for de novo design. Usually, natural proteins incorporate two or more (hetero- or homo-) metal ions into their frameworks to perform their functions, but the design of *multiple* metal-binding sites is usually difficult to achieve. Here, we undertook the de novo engineering of heterometal-binding sites, Ni(II) and Cu(II), into a designed coiled coil structure based on an isoleucine zipper (IZ) peptide. Previously, we described two peptides, IZ-3adH and IZ-3aH. The former has two His residues and forms a triple-stranded coiled coil after binding Ni(II), Zn(II), or Cu(II). The latter has one His residue, which allowed binding with Cu(II) and Zn(II), but not with Ni(II). On the basis of these properties, we newly designed IZ(5)-2a3adH as a heterometal-binding peptide. This peptide can bind Cu(II) and Ni(II) simultaneously in the hydrophobic core of the triple-stranded coiled coil. The first metal ion binding induced the folding of the peptide into the triple-stranded coiled coil, thereby promoting the second metal ion binding. This is the first example of a peptide that can bind two different metal ions. This construction should provide valuable insights for the de novo design of metalloproteins.

## Introduction

Studies on de novo designed proteins include the construction of unique tertiary structures and the creation of new functions. Thus far, many types of secondary and tertiary structures, such as α-helical bundle and coiled coil,[1] collagen,[2] β-sheet protein,[3] and α/β-protein,[4] have been designed and successfully con-structed. However, as compared to structural design, only few examples of functional design have been reported. Metal ions serve a number of roles in protein folding,[5] enzymatic reactions,[6] electron-transfer processes,[7] and so on. Metal binding is, therefore, one of the most attractive targets in de novo design. Proteins usually require specific metal ions for their structural formation and functions. For example, the zinc finger proteins and the *Escherichia coli* Ada protein require Zn(II) for a proper folding and ligand recognition.[8] Hg(II) is responsible for the genetic switch of MerR, a metalloregulatory protein.[5a,9] Some proteins possess two metal ions. Diiron proteins engage in the reversible binding of oxygen, electron transfer, and catalysis of hydroxylation reactions.[10] Two metal ions, Cu(II) and Zn(II), are responsible for the function of superoxide dismutase.[11]

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In de novo design, most metal ion-binding sites are engineered outside the protein structures.[12] In a few examples, a conformational change occurred after the metal binding. Designed zinc finger peptides folded after the binding of Zn(II) to His and Cys residues.[13] Binding of lanthanide ions to γ-glutamic acids in the designed peptide induced the folding of a double-stranded coiled coil.[14] Within a hydrophobic pocket of de novo designed proteins, Ni(II) bound to six His residues, and Hg(II) and Cd(II) bound to three Cys residues in the triple-stranded coiled coil.[15] Two Zn(II) binding sites were also created using Glu-Xxx-Xxx-His sequences in a four-helix bundle protein.[16] However, different two-metal ion-binding sites have not been constructed thus far, and they should be an attractive target to further the de novo design of functional proteins.

Here, a heterometal ion-binding site was designed in the hydrophobic core of a triple-stranded α-helical coiled coil structure. The α-helical coiled coil has the representative amino acid sequence of (*defgabc*)$_n$ heptad repeats.[17] The *a* and *d* positions are usually occupied by hydrophobic residues, which form the hydrophobic core. We previously synthesized the de novo designed peptide, isoleucine zipper (IZ), [YGG(IEKKIEA)₄] (*defgabc*), which forms a parallel triple-stranded α-helical coiled coil structure.[18] Starting from this peptide, we searched for proper mutations for IZ sequences that could regulate the assembly of the peptide by metal ions. The IZ-3adH peptide, which possesses two His residues at the *d* and *a* positions of the third heptad repeat, binds Ni(II), Zn(II), or Cu(II) using the two His residues and forms a triple-stranded coiled coil.[15a] On the other hand, the IZ-3aH peptide, which has only one His residue, binds Zn(II) and Cu(II), but not Ni(II).[19] On the basis of these observations, we designed the peptide IZ(5)-2a3adH, composed of 38 amino acid residues including three His residues. This peptide can bind Ni(II) and Cu(II) simultaneously in the hydrophobic core of the triple-stranded α-helical coiled coil.

### Results

**Design of Two-Metal Binding Sites.** We previously prepared the de novo designed peptide, IZ, [YGG(IEKKIEA)₄] (*defgabc*), which forms a parallel triple-stranded α-helical coiled coil

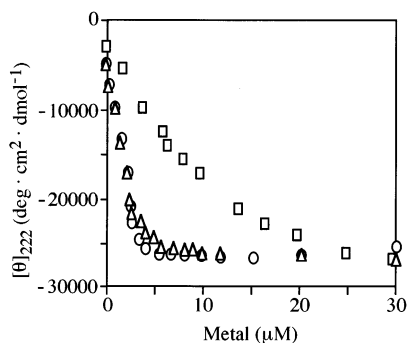|  | 1 | 2 | 3 | 4 | 5 |  |
|---|---|---|---|---|---|---|
|  | *defgabc* | *defgabc* | *defgabc* | *defgabc* | *defgabc* |  |
| IZ | YGG | IEKKIEA | IEKKIEA | IEKKIEA | IEKKIEA |  |
| IZ-3adH | --- | ------- | ------- | H---H-- | ------- |  |
| IZ-3aH | --- | ------- | ------- | ----H-- | ------- |  |
| IZ-2a3adH | --- | ------- | ----H-- | H---H-- | ------- |  |
| IZ(5)-2a3adH | --- | ------- | ----H-- | H---H-- | ------- | IEKKIEA |

**Figure 1.** Amino acid sequences of IZ, IZ-3adH, IZ-3aH, IZ-2a3adH, and IZ(5)-2a3adH. Only the amino acids that differ from those in the IZ sequence are indicated. Bars indicate the same amino acids as in IZ. The heptad numbers and positions are indicated above the IZ sequence.
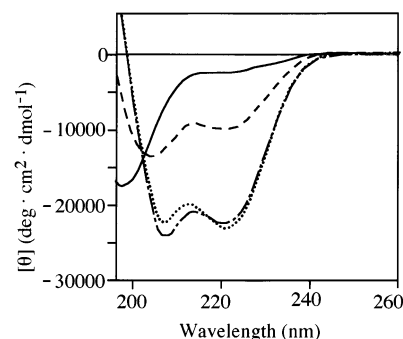


**Figure 2.** Circular dichroism spectra of the IZ(5)-2a3adH peptide in the absence (solid line) and presence of metal ions (ZnCl₂, broken line; CuCl₂, dotted line; NiCl₂, dash−dotted line). The measurements were performed in 10 mM sodium phosphate containing 0.1 M NaCl (pH 7.6) at 20 °C. The peptide and metal concentrations were 20 and 6.7 $\mu$M, respectively.

structure (Figure 1). On the basis of IZ, we have designed two transition-metal ion-binding peptides, IZ-3adH and IZ-3aH. These have two or one His residues, respectively, in the 30 amino acid residue peptides, and they exhibited different metal ion selectivity. On the basis of the information obtained from these peptides, we designed a two-metal binding peptide. The metal ions can favorably coordinate to two His residues at the *i* and *i* + 4 positions, rather than those at the *i* and *i* + 3 positions. The His residues at the *3d* and *3a* positions are separated by three amino acids, while the *2a* and *3d* positions are separated by two amino acids. Consequently, the metal binding is more favorable between the *3d* and *3a* positions. Therefore, the first metal-binding site should be the *3d* and *3a* positions, and the *2a* position would become the second metal-binding site. Thus, the first metal ion binding to the two His residues at the *3d* and *3a* positions is expected to induce the folding, and then the second metal ion can bind to the *2a* position. We designed the IZ(5)-2a3adH peptide, which has three His residues at the hydrophobic positions of the continuous layer (Figure 1). First, we designed IZ-2a3adH, consisting of four heptad repeats, but the His residues in the hydrophobic core destabilized the coiled coil structure, even after the complexation with metal ions. Hence, the IZ(5)-2a3adH peptide, with five heptad repeats, was designed to increase the structural stability.

**Characterization of the IZ(5)-2a3adH Peptide. Circular Dichroism Spectroscopy.** Circular dichroism (CD) spectroscopy was performed to characterize the secondary structure of the designed peptide. The IZ(5)-2a3adH peptide exhibited a minimum below 200 nm, which is characteristic for a random coil structure, as shown in Figure 2. To analyze the effect of metal ions on the structure of this peptide, 1/3 equiv of metal ions
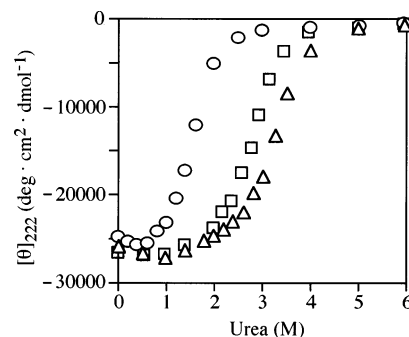
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**Figure 3.** Ni(II) (△), Cu(II) (○), and Zn(II) (□) titration profiles of IZ(5)-2a3adH (15 $\mu$M) measured by CD spectroscopy at 20 °C and pH 7.6. The $[\theta]_{222}$ values were monitored and plotted as a function of the metal ion concentration.



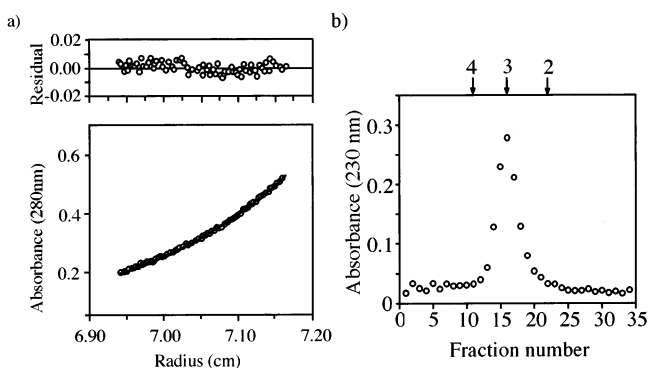**Figure 4.** Urea denaturation curves for IZ(5)-2a3adH in the presence of Cu(II) (○), Ni(II) (□), and Cu(II) and Ni(II) (△). The denaturation curves were recorded in 10 mM sodium phosphate at pH 7.0 with peptide and metal concentrations of 20 and 6.7 $\mu$M, respectively. Ni(II) (6.7 $\mu$M) and Cu(II) (6.7 $\mu$M) were both present for the binding of two metals.



**Figure 5.** (a) Representative analytical ultracentrifugation sedimentation data for the IZ(5)-2a3adH−Ni(II) complex. The concentrations of the peptide and the metal were 100 and 500 $\mu$M, respectively. The sample was centrifuged at 32 000 rpm at 25 °C. (b) Gel filtration analysis of a mixture of IZ(5)-2a3adH (20 $\mu$M) and NiCl$_2$ (100 $\mu$M). The numbers 4, 3, and 2 at the top indicate the places where the peptide standards were eluted.

(6.7 $\mu$M) were added to the peptide sample (20 $\mu$M). In the presence of Ni(II) and Cu(II), this peptide exhibited a typical α-helical structure, with minima at 208 and 222 nm. The $[\theta]_{222}$ to $[\theta]_{208}$ ratios of the CD spectrum in the presence of Cu(II) and Ni(II) were 1.04 and 0.93, respectively. The peptide binding with Cu(II) probably formed an α-helical coiled coil structure, while a partially perturbed α-helical coiled coil structure was induced by Ni(II). However, Zn(II) did not induce the α-helical structure as efficiently as Ni(II) and Cu(II). Upon the further addition of Zn(II) (up to 20−30 equiv vs peptide), the α-helical structure was induced, as shown in Figure 3. These results showed that the coordination of Ni(II) and Cu(II) to the His residues was more preferable than that of Zn(II). In the case of IZ-3adH, Cu(II) can bind and induce an α-helical structure; however, Cu(II) showed an abnormal behavior. With the addition of Cu(II), an α-helical structure was induced, but the α-helical content was decreased because of the favorable monomeric structure in the presence of excess Cu(II). On the other hand, even with the further addition of Cu(II) up to 40 equiv to IZ(5)-2a3adH, the α-helical content was maintained, while the α-helical content of the IZ-3adH peptide was reduced to 50% under the same conditions.[15a] This result suggests that another Cu(II) may have been bound to the second metal-binding site following the first metal binding to IZ(5)-2a3adH in the presence of excess Cu(II).

Ni(II) binds most efficiently to IZ-3adH as compared to other metal ions; however, Ni(II) is ineffective with IZ-3aH. On the other hand, Cu(II) binds to both IZ-3adH and IZ-3aH. These results suggest that Ni(II) binds to the His residues at the *3a* and *3d* positions of IZ(5)-2a3adH, and the remaining His residue at the *2a* position is free. Then Cu(II) binds to the remaining His residue at the *2a* position. To analyze the effect of the heterometal ion binding, we measured the stability of the coiled coil structure toward urea denaturation in the presence of Cu(II) and Ni(II) or a mixture of Cu(II) and Ni(II) (Figure 4). The denaturation midpoints in the presence of Ni(II) or Cu(II) alone were 2.5 and 1.7 M, respectively, showing that Ni(II) was more effective than Cu(II). In the presence of a mixture of Ni(II) and Cu(II), the midpoint was increased to 3.2 M, suggesting that the peptide bound both Ni(II) and Cu(II).

**Oligomerization of the Peptide.** The oligomerization states of the Ni(II)−IZ(5)-2a3adH complex were determined by a sedimentation equilibrium centrifugation analysis (Figure 5a). The data for the metal−peptide complex were fitted to a single species. The residuals are random and centered around zero,
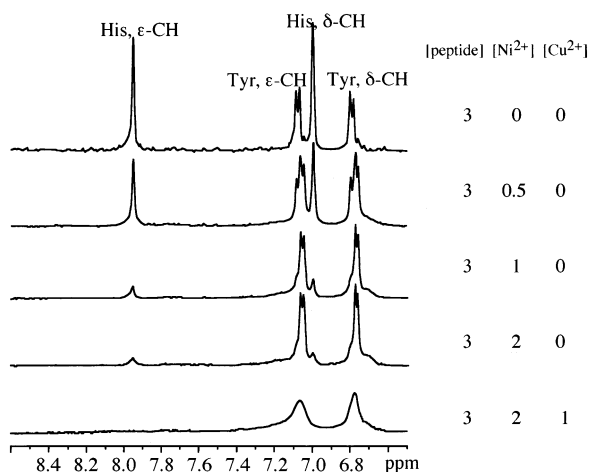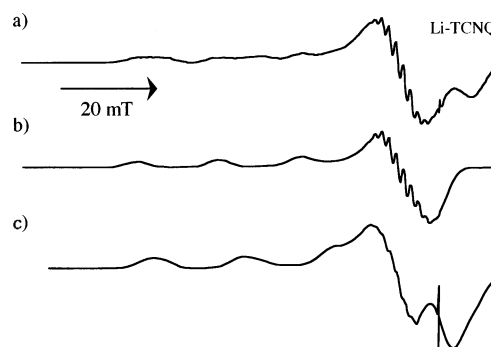
indicating that the complex is a single homogeneous species. The apparent molecular size of the complex is 13 300 ± 50, indicating that the Ni(II)−IZ(5)-2a3adH complex is trimerized (the calculated molecular mass for the trimerized peptide is 13 279 Da). The apparent MW for the Cu(II)−IZ(5)-2a3adH complex could not be obtained because of the influence of the Cu(II) complex absorption. Therefore, the Cu(II), Cu(II)− and Ni(II)−peptide complexes were analyzed by the size exclusion chromatography using Sephadex G-50. The coiled coil has an extended structure, and hence, we used peptides that have lengths similar to IZ(5)-2a3adH and form the coiled coil structures, as the standard dimer, trimer, and tetramer references for the analyses by size exclusion chromatography. The IZ(5)-2a3adH peptide in the presence of Cu(II) or Ni(II) and Cu(II) was eluted at the same fraction numbers as the Ni(II)−peptide complex and the trimeric coiled coil standard (Figure 5b). These results indicate that the IZ(5)-2a3adH peptide is trimerized in the presence of either Ni(II) alone, Cu(II) alone, or a mixture of Ni(II) and Cu(II).

**Nuclear Magnetic Resonance (NMR) Measurements.** To assess the binding of the metal ions to IZ(5)-2a3adH, we performed an NMR study. In the absence of Ni(II), aromatic protons from the Tyr residue appeared at 6.80 and 7.09 ppm, and His signals were at 7.00 and 7.95 ppm (Figure 6). These chemical shifts are typical in a random coil structure, and the imidazole ring proton signals from the three His residues were degenerated. Upon the addition of Ni(II), the aromatic protons

His, ε-CH          His, δ-CH

Tyr, ε-CH    Tyr, δ-CH

[peptide] [Ni²⁺] [Cu²⁺]

| | | |
|---|---|---|
| 3 | 0 | 0 |
| 3 | 0.5 | 0 |
| 3 | 1 | 0 |
| 3 | 2 | 0 |
| 3 | 2 | 1 |

8.4  8.2  8.0  7.8  7.6  7.4  7.2  7.0  6.8  ppm

**Figure 6.** One-dimensional $^1$H NMR spectra of the IZ(5)-2a3adH. Measurements were performed in $^2$H$_2$O (pH 6.5) at the peptide concentration of 0.2 mM, corresponding to 0.067 mM of the trimerized IZ(5)-2a3adH. The ratios of peptide, Ni(II), and Cu(II) are indicated in the figure. The spectrum in the absence of metal ion was assigned as follows: 6.80 ppm (tyr, δ-CH), 7.00 (His, δ-CH), 7.09 (Tyr, ε-CH), 7.95 (His, ε-CH).

from the Tyr residues were shifted to high field at 6.76 and 7.05 ppm, respectively. The chemical shift change of the aromatic protons ended at the IZ(5)-2a3adH and Ni(II) ratio of 3:1, indicating that three IZ(5)-2a3adH peptides bind one Ni(II). The high field shift of the aromatic protons was observed during the folding of IZ-3adH into the triple-stranded coiled coil by the addition of Ni(II).[15a] Following the addition of Ni(II), the imidazole protons from the His residues were gradually shortened. Binding of the paramagnetic Ni(II) ion leads to perturbations in the line widths and chemical shifts of the NMR resonances of a peptide ligand.[20] Both effects are dependent on the distance from the nucleus to the metal ion ($1/r^6$ and $1/r^3$ dependence, respectively). In the case of IZ(5)-2a3adH, one Ni(II) was designed to bind to the His residues at the *3a* and *3d* positions. Although the imidazole protons completely disappeared in the IZ-3adH peptide at the peptide−Ni(II) ratio of 3:1, they were still observed even upon the further addition of Ni(II) to the ratio of 3:2. It is considered that the His residue at the *2a* position is free and close to the Ni(II) bound to the His residues at the *3d* and *3a* positions. As a consequence, the imidazole protons were not completely shielded by Ni(II) and were partially affected. This result also indicates that the change of the imidazole ring proton was affected by the Ni(II) bound to the peptide, but not by the Ni(II) surrounding the peptide in solution. Because of the strong paramagnetic nature of Cu(II), the subsequent addition of Cu(II) to the peptide−Cu(II) ratio of 3:1 caused the remaining imidazole protons to completely disappear, suggesting that Cu(II) bound to the His residue at the *2a* position. Although the aromatic protons from the Tyr residue were broadened, they were still observed at the same positions. It is reasonable that the Cu(II) bound to the His residue at the *2a* position affects the Tyr residue at the amino terminus through space. Cu(II) binding to metalloproteins is known to induce severe line broadening of a protein signal as compared to Ni(II), because the electron-relaxation time ($T_s$) of Cu(II) is a 1−3 orders faster.[21]

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a)

20 mT →                                    Li-TCNQ

b)

c)

**Figure 7.** ESR spectra recorded at 77 K for frozen solutions of (a) IZ(5)-2a3adH−Cu(II) complex, (b) IZ-2adH−Cu(II) complex, and (c) Ni(II)−Cu(II)−IZ(5)-2a3adH complex prepared at pH 6.6. The final concentrations of Cu(II) ion and IZ(5)-2a3adH, Cu(II) ion and IZ-3adH, and Ni(II) and Cu(II) ions and IZ(5)-2a3adH in the 100 mM NaCl solution were 0.3 and 0.9 mM, 0.3 and 0.9 mM, and 0.3, 0.3 and 0.9 mM, respectively. The pH of the solution was adjusted using 0.01 N NaOH and 0.01 N HCl while monitoring the pH. lithium salt of tetracyanoquinodimethane, Li-TCNQ, is an external standard ($g = 2.0025$).

**Electron Spin Resonance (ESR) Measurements.** ESR analyses were carried out for the IZ(5)-2a3adH−Cu(II) and Ni(II)−Cu(II)−IZ(5)-2a3adH complexes to obtain information about the coordination structure. Each reaction solution, composed of IZ(5)-2a3adH (0.9 mM) and CuCl$_2$ (0.3 mM) or NiCl$_2$ (0.3 mM) and CuCl$_2$ (0.3 mM) prepared at pH 6.6, was subjected to ESR measurements. As shown in Figure 7a, the reaction solution of IZ(5)-2a3adH and CuCl$_2$ (0.3 mM) generated a complicated ESR line shape composed of at least three copper complexes with different ESR parameters. The super hyperfine splitting (nine lines; aN = 1.53 mT) in the $g_\perp$ region is characteristic of the copper complex bearing four imidazole nitrogen atoms at the equatorial positions. The apparent $g_\perp$ and aN values of this species are consistent with those of the Cu(II)−IZ-3adH complex ($g_{||} = 2.268$, $A_{||} = 17.3$ mT, $g_\perp = 2.061$, and aN = 1.53 mT), as depicted in Figure 7b. A comparison of the ESR line shape in the $g_{||}$ region of these two complexes (Figure 7a,b) clearly revealed hyperfine splitting, similar to that of the Cu(II)−IZ-3adH complex, in the ESR signal of the Cu(II)−IZ(5)-2a3adH complex (Figure 7a). This suggests that the copper complex bound to the imidazoles at the *3a* and *3d* positions of IZ(5)-2a3adH peptide is the major species. The further addition of CuCl$_2$ did not affect the ESR spectrum of the Cu(II)−IZ(5)-2a3adH complex. As shown in Figure 7c, the ESR spectrum of the Ni(II)−Cu(II)−IZ(5)-2a3adH complex revealed an ESR line shape derived from a single Cu(II) complex, as featured by the following ESR parameters: $g_{||} = 2.210$, $A_{||} = 18.3$ mT, $g_\perp = 2.048$. The seven lines of super hyperfine splitting observed in the $g_\perp$ region supported the ligation of three nitrogen atoms to the Cu(II) ion. The ESR parameters were similar to those of the IZ-3aH−Cu(II) complex,[19] indicating that the Cu(II) ion engages three nitrogens of the His residues and an oxygen donor, possibly derived from H$_2$O. In addition, the ESR signal of this complex was also involved in the complicated ESR signal recorded for the mixture of IZ(5)-2a3adH and CuCl$_2$ (Figure 7a). Even after this sample was annealed, the identical ESR spectrum was still obtained. When the final concentration of CuCl$_2$ was increased to 0.6 mM, the ESR signal of the nonspecifically bound Cu(II)−peptide adduct ($g_{||} = 2.235$, $A_{||} = 18.7$ mT, $g_\perp = 2.048$) was observed together with that of the Ni(II)−Cu(II)−IZ(5)-2a3adH complex (data not shown). A
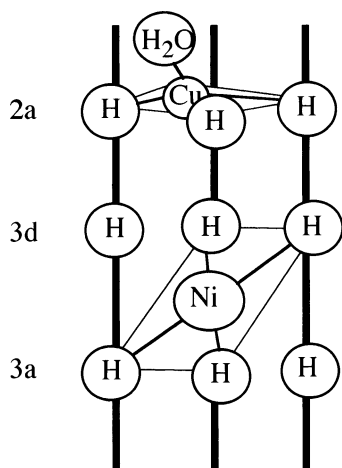
**Figure 8.** Structural model of the Ni(II),Cu(II)−peptide complex, based on ESR analyses. One of the possible coordination structures is depicted. Since the ESR of the Ni(II) ion was silent even at 4.2 K, its spin state is postulated to be low-spin ($S = 0$). The low-spin Ni(II) ions prefer the square-planar coordination geometry, consistent with His residues at the *3a* and *3d* positions, by analogy with the case of the Ni(II)−IZ-3adH complex. Cu(II) bound to the planar three His residues at the *2a* positions and water.[19] This might cause structural perturbations around the metal-binding sites. The three vertical bold bars indicate the α-helix.

similar, nonspecific Cu(II)−peptide complex was also observed upon the addition of CuCl$_2$ (0.3mM) to the solution of the Ni-(II)−IZ-3adH complex. This supports the idea that the Ni(II) ion bound to imidazole at the *a* and *d*positions of IZ(5)-2a3adH or IZ-2adH was not replaced, even in the presence of an excess amount of the Cu(II) ion. These observations demonstrate that the coordination of Ni(II) at *3a* and *3d*positions regulates the coordination mode of Cu(II) in the coiled coil structure of the IZ(5)-2a3adH peptide.

## Discussion

The design of metal-binding sites in proteins provides important insights related to understanding the biological functions of metal ions in natural proteins, improving the design principles, and creating new functions.[22] Although many metal-binding sites have been designed, most of them were placed at the solvent exposed sites of natural or designed proteins. However, the metal ions in natural proteins are usually not completely exposed to the solvent. Only few examples of metal-binding sites in hydrophobic positions have been reported, by DeGrado,[16] Pecoraro,[15d] and us.[15b] We previously described three examples with different metal ion selectivities.[15a,b,19] We combined two design procedures to create one peptide and succeeded in placing two different metal ions in the vicinity of a hydrophobic position. A possible coordination structure of the Ni(II)−Cu(II)−IZ(5)-2a3adH complex is illustrated in Figure 8. This construction is a starting point for the design of metalloproteins with efficient functions that are similar to those of natural proteins.

Considering the two-metal binding sites in the peptide, we prepared the IZ(5)-2a3adH peptide, where three His residues were placed in spatial vicinity of each other. The metal coordination is known to be more favorable at the *i* and *i* + 4 positions than at the *i* and *i* + 3 positions.[23] The His residues

at the *3a* and *3d* positions are situated at the *i* and *i* + 4 positions, while the *2a* and *3d* positions are situated at the *i* and *i* + 3 positions. The first metal-binding site is, therefore, the two His residues at the *3a* and *3d* positions. The second metal-binding site is the His residue at the *2a* position. We demonstrated the binding of two metal ions, Ni(II) and Cu(II), to IZ(5)-2a3adH by CD and NMR spectroscopies. From the ESR study, Cu(II) apparently bound to the three planer His residues. Ni(II) probably interacted with the rest of the His residues. It is reasonable to consider that Ni(II) bound to the His residues at the *3a* and *3d* positions, and Cu(II) at the *2a* position. Since Ni(II) was the most effective divalent ion for the IZ-3adH peptides, as compared to Cu(II) and Zn(II), the square-planar geometry of the Ni(II) complex might be supported by six His residues at *3a* and *3d* positions. In fact, the Ni(II) ion did not bind to the IZ-3aH peptide. On the other hand, Cu(II) effectively bound to both peptides. Thus, we attained selectivity for two metal ions in the coiled coil structure. We are certain that Cu(II) bound at the *3a* position, and Ni(II) at the *2a* and *3d* positions. However, Ni(II) also did not induce the helical conformation of IZ-2a3dH, which has two His residues at the *2a* and *3d* positions (data not shown). We have no evidence that Ni(II) and Cu(II) exchange occurred, although further analyses might be necessary. In the IZ(5)-2a3adH peptide, the first metal-binding function in structural formation and the second metal binding serve to increase the structural stability.

In the case of the IZ-3adH peptide, the excess Cu(II) stabilized the monomeric form because of the strong coordination of Cu(II) to the two His residues at the *i* and *i* + 4 positions of the same strand, rather than the formation of a trimeric coiled coil. The peptide formed the α-helical conformation around the Cu(II)-binding site, and the rest of the peptide assumed a flexible conformation. This led to a decrease in the α-helicity of IZ-3adH in the prescence of more than 100 $\mu$M Cu(II).[15a] On the other hand, the IZ(5)-2a3adH peptide maintained the α-helical content, even 500 $\mu$M Cu(II) (data not shown). It was suggested that the IZ(5)-2a3adH peptide bound two Cu(II). Contrary to our expectations, however, the ESR study indicated that one Cu(II) bound in three different manners in the hydrophobic core. The arrangement of two Cu(II) in the vicinity of the hydrophobic core of the coiled coil can be accomplished by analyzing the precise coordination of Cu(II) in the IZ(5)-2a3adH peptide. This construction could be a model for catechol oxidase and plastocyanine.

Here, we have demonstrated that the peptides bound two different metal ions and underwent homotrimerization. This is the first example of two different metal ions binding in proteins. We used the His residues only as ligands for the metal ions in this report. By selecting the species and the positions of the amino acid ligands for the metal ions, it will be possible to apply these findings to design various metal ion-binding combinations. We previously reported the constructions of AAB- and ABC-type heterotrimeric coiled coils.[24] Using the hetero-trimers, we can extend our designs not only to mimic precisely the various metal-binding sites of natural proteins but also to create new metal-binding sites. These studies should lead to a

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better understanding of the roles of metal ions in natural proteins. They will also facilitate further applications for new structural formations and catalytic functions.

## Experimental Section

**Peptide Synthesis and Purification.** The IZ(5)-2a3adH peptide was synthesized on an Applied Biosystems model 433A automated synthesizer, using Rink amide resin (substitution 0.37 mmol/g), based on the standard Fastmoc 0.1 mmol protocol. The side chain protection groups were: Glu(OtBu), Lys(Boc), His(Trt), and Tyr(tBu). Fmoc-amino acid derivatives (1 mmol) were coupled to the resin after activation by HBTU/HOBt. Deprotection of the side chain and cleavage from the resin were performed by treatment with TFA containing 2.5% ethanedithiol and 2.5% $H_2O$ (v/v) at room temperature for 1.5 h. Purification was carried out by reverse-phase HPLC on a YMC-Pack ODS-A column (10 mm i.d. × 250 mm, 5 $\mu$m, YMC Inc., Japan) with a linear gradient of 25−35% $CH_3CN/H_2O$ containing 0.1% TFA over the course of 30 min. The final product was characterized by analytical HPLC and was confirmed by MALDI-TOF mass spectrometry, *m/z*: 4427.6 (calcd, 4426.1) for IZ(5)-2a3adH.

**Circular Dichroism Spectroscopy.** CD measurements were performed on a Jasco-820 spectropolarimeter, using a 2-mm cuvette at 20 °C. The peptide concentration was determined by measuring the tyrosine absorbance in 6 M guanidium chloride, using $\epsilon_{275} = 1450$ $M^{-1}$ $cm^{-1}$.[25] The mean residue ellipticity, $[\theta]$, is given in units of deg·cm²·dmol⁻¹. CD spectra were obtained in 20 mM phosphate buffer (pH 7.6) at a peptide concentration of 20 $\mu$M, in the absence and presence of 20 $\mu$M Zn(II), Ni(II), and Cu(II). Metal ion titration was carried out in the same buffer by monitoring $[\theta]_{222}$ as a function of the metal concentration, which ranged from 1 to 800 $\mu$M for IZ(5)-2a3adH. The peptide concentration was 15−30 $\mu$M.

Thermal transition curves were obtained by monitoring $[\theta]_{222}$ as a function of temperature with a 2-mm path length cuvette. The total peptide concentration was 20 $\mu$M, and the temperature was increased at a rate of 1 °C/min.

**Sedimentation Equilibrium.** Sedimentation equilibrium analysis was carried out with a Beckman XL-I Optima Analytical Ultracentrifuge equipped with absorbance optics. The peptide concentrations were 20 and 100 $\mu$M in phosphate buffer (20 mM, pH 7.6) containing 5 equiv of $NiCl_2$. The samples were independently rotated at 25 000 rpm at 20 °C for 20 h and were monitored at a wavelength of 280 nm. The apparent molecular weight was obtained by fitting the data to a single ideal species without considering any influence of $ZnCl_2$, using Origin Sedimentation Single Data Set Analysis (Beckman). A partial specific volume of 0.756 mL/g was calculated for IZ(5)-2a3adH, using the method of Cohn and Edsall.[26]

**Size Exclusion Chromatography.** Mixtures of IZ(5)-2a3adH (10 $\mu$M − 1 mM) with 5 equiv of $NiCl_2$ and IZ(5)-2a3aH (20 $\mu$M) with $CuCl_2$ (10 $\mu$M) or with $NiCl_2$ (10 $\mu$M) and $CuCl_2$ (5 $\mu$M) were each dissolved in 0.2 mL of 10 mM sodium phosphate buffer at pH 7.0. The samples were applied on Sephadex G-50 (0.6 cm (i.d.) × 9 cm) and were eluted with the same buffer at pH 7.0. The fractions (90 $\mu$L) were collected and monitored at a wavelength of 230 nm. As the peptide standards, GCN4-pLI,[27] IZ,[18] and GCN4-p1[27] were used for tetramer, trimer, and dimer, respectively.

**Nuclear Magnetic Resonance.** NMR spectroscopy was performed on a Bruker DRX500 spectrometer operated at 500.03 MHz for ¹H. Chemical shifts were referenced internally to 0 ppm with trimethyl-silylpropionic acid. One-dimensional spectra were measured at 25 °C with suppression of the residual water signal by weak presaturation. The data sets were defined by 8 k complex points, and 32 scans were accumulated using a spectral width of 8289.3 Hz. Samples were prepared at an approximate concentration of 0.2 mM in ²$H_2O$ containing 50 mM sodium phosphate buffer (pH 6.5, direct meter reading). In the metal ion titration study, small aliquots of an $NiSO_4$ solution were added to the peptide solution. After the $NiSO_4$ was added, small aliquots of $CuSO_4$ were added. The total volume change of the solution was about 10% over the titration.

**ESR Analysis.** ESR measurements were carried out at 77 K using a JEOL-TE300 spectrometer, equipped with 100 kHz field modulation (0.63 mT). The magnetic field strength was calibrated by hyperfine splitting (8.69 mT) of Mn(II) ion doped in Mg(II)O powder, as an external standard. The *g* and *A* values were estimated by means of computer simulation of spectra after trial-and-error curve-fitting analysis. The mixture of peptide (0.9 mM) and Cu(II) (0.3 mM) or $NiCl_2$ (0.3 mM) and $CuCl_2$ (0.3 mM) was prepared in 100 mM NaCl, and the pH of the solution was adjusted using 0.01 N NaOH and 0.01 N HCl while monitoring the pH.

**Supporting Information Available:** Some experimental data (size exclusion chromatography and sedimentation equilibrium analyses). This material is available free of charge via the Internet at http://pubs.acs.org.

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