Protein Science (2000), *9*:1327–1333. Cambridge University Press. Printed in the USA.

Soft metal ions, Cd(II) and Hg(II), induce triple-stranded α-helical assembly and folding of a de novo designed peptide in their trigonal geometries

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(RECEIVED August 10, 1999; FINAL REVISION February 16, 2000; ACCEPTED April 27, 2000)

Abstract

We previously reported the de novo design of an amphiphilic peptide [YGG(IEKKIEA)$_4$] that forms a native-like, parallel triple-stranded coiled coil. Starting from this peptide, we sought to regulate the assembly of the peptide by a metal ion. The replacement of the Ile18 and Ile22 residues with Ala and Cys residues, respectively, in the hydrophobic positions disrupted of the triple-stranded α-helix structure. The addition of Cd(II), however, resulted in the reconstitution of the triple-stranded α-helix bundle, as revealed by circular dichroism (CD) spectroscopy and sedimentation equilibrium analysis. By titration with metal ions and monitoring the change in the intensity of the CD spectra at 222 nm, the dissociation constant K_d was determined to be 1.5 ± 0.8 μM for Cd(II). The triple-stranded complex formed by the ^{113}Cd(II) ion showed a single ^{113}Cd NMR resonance at 572 ppm whose chemical shift was not affected by the presence of Cl^- ions. The ^{113}Cd NMR resonance was connected with the βH protons of the cysteine residue by ^{1}H–^{113}Cd heteronuclear multiple quantum correlation spectroscopy. These NMR results indicate that the three cysteine residues are coordinated to the cadmium ion in a trigonal-planar complex. Hg(II) also induced the assembly of the peptide into a triple-stranded α-helical bundle below the Hg(II)/peptide ratio of 1/3. With excess Hg(II), however, the α-helicity of the peptide was decreased, with the change of the Hg(II) coordination state from three to two. Combining this construct with other functional domains should facilitate the production of artificial proteins with functions controlled by metal ions.

Keywords: coiled coil; de novo design; folding; helical structures; metalloproteins

Studies on de novo designed proteins involve the construction of proteins with unique tertiary structures and the creation of new functional proteins. The coiled coil structure is often observed in natural proteins for the intermolecular assemblies of the functional domains. This motif, due to its structural simplicity and biomolecular significance, has been the subject of extensive analyses to understand the principles of de novo design, as well as protein folding and stability (Lau et al., 1984; O'Neil & DeGrado, 1990; Harbury et al., 1993). Furthermore, the designed coiled coils can be fused to various functional peptides or domains of natural proteins for biological and medical applications (Pack & Plückthun, 1992; Hodges, 1996; Terskikh et al., 1997).

The designed coiled coil, which drastically changes its conformation depending on external stimuli, should be useful to control the associations and the functions of domains attached to the peptide. Among the various external stimuli, metal binding has been studied extensively, and the factors required for metal binding are well understood. A variety of metal binding sites have been designed, but they are on the surfaces of preformed artificial supramolecules, such as an α-helical bundle protein (Handel & DeGrado, 1990; Regan & Clarke, 1990; Regan, 1995; Dieckmann et al., 1997, 1998), a β-sheet protein (Pessi et al., 1993), and an α/β protein (Klemba et al., 1995). Accordingly, the metal binding stabilizes their structures but is not involved in the process of their folding or assembly.

There are several examples of de novo designed α-helical bundle structures induced by metal ions. However, in most cases, pendant molecules, such as a bipyridyl group, were attached to the peptides, and the binding of the metal ions to the pendant mol-

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Abbreviations: MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; rink amide resin, 4-(2′, 4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin; Fmoc, 9-fluorenylmethyloxycarbonyl; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; OtBu, *tert*-butyl ester; Boc, *tert*-butoxycarbonyl; Trt, trityl; TFA, trifluoroacetic acid; CD, circular dichroism; HPLC, high-performance liquid chromatography; HMQC, heteronuclear multiple quantum coherence; UV, ultraviolet.

ecules induced the assembly and the folding of the peptides (Lieberman & Sasaki, 1991; Ghadiri et al., 1992a, 1992b; Ghadiri & Case, 1993). Kohn et al. (1998) reported the metal-induced folding of a double-stranded coiled coil. The folding was initiated by the binding of lanthanides, such as La(III), to γ-carboxyglutamic acids, Gla, at the solvent exposed sites. The lanthanides belong to the "hard" acid category according to the Lewis acid-base theory (Glusker, 1991).

Based on peptides described previously by Harbury et al. (1993) and O'Neil and DeGrado (1990), we prepared an amphiphilic 31-residue peptide, IZ (Fig. 1), that forms a parallel triple-stranded coiled coil with native-like folding properties in solution (Suzuki et al., 1998b). A metal binding site could be engineered in the hydrophobic core of IZ. Two His residues were introduced in the hydrophobic positions of IZ (IZ-3adH, Fig. 1) (Suzuki et al., 1998a). IZ-3adH efficiently bound a Ni^{2+} ion, which is a "medium" metal ion, and folded into a triple-stranded α-helical bundle.

As an example of a "soft" metal ion, a Hg(II) binding site has been successfully engineered in the hydrophobic positions of two- and three-helical bundles (Dieckmann et al., 1997, 1998). The Hg(II) bound to cysteine residues and stabilized the helical bundle structures. In natural proteins, metallothioneins are cysteine rich proteins, composed of approximately 60 amino acid residues, that bind multiple metals in metal-thiolate clusters (Kägi & Kojima, 1987; Fowle & Stillman, 1997). MerR is a metalloregulatory protein that functions as an Hg(II)-responsive genetic switch (Frantz & O'Halloran, 1990; Ansari et al., 1995). These proteins bind a variety of "soft" metal ions, including Hg(II), Cd(II), and Zn(II), using Cys residues (Kägi & Kojima, 1987; Kägi & Schäffer, 1988; Ralston & O'Halloran, 1990; Watton et al., 1990). To confer the "soft" metal ion selectivity to our triple-stranded coiled coil, we substituted Ala and Cys residues for the two Ile residues at the d and a positions, respectively, of the third heptad repeat of IZ. This newly designed peptide, IZ-AC, was selectively induced into triple stranded coiled coil by "soft" metal ions.

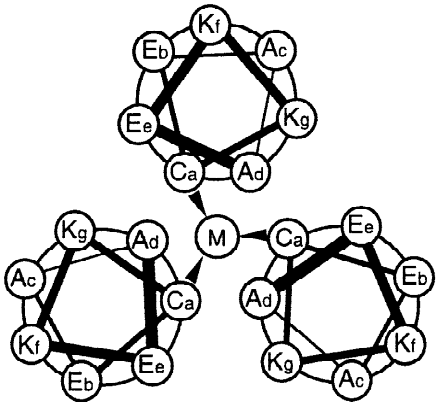

Fig. 1. Helical wheel representation of the third heptad of IZ-AC in the parallel orientation, viewed from the N- to the C-terminus. The sequences of IZ, IZ-3adH, and IZ-AC are also represented. The four heptad repeats are preceded by the YGG sequence for the peptide quantitation.

Results

We previously prepared a parallel triple-stranded coiled coil, IZ, with an amino acid sequence containing four heptad repeats, $YGG(IEKKIEA)_4$ (Suzuki et al., 1998b). Two Ile residues, at the d and a positions of the third heptad repeat of IZ, were substituted with Ala and Cys residues, respectively, and this peptide was designated as IZ-AC (Fig. 1). Although the Ala residue has a high α-helical propensity, it destabilizes the coiled coil structure when it is in the hydrophobic core, due to the small size of its side chain (Monera et al., 1994). The reduced Cys residue also destabilizes the coiled coil structure (Moitra et al., 1997). Thus, the CD spectrum of the IZ-AC peptide showed a random structure with a minimum at 200 nm at pH 7.6 and 20 °C (Fig. 2). Size exclusion chromatography using Sephadex G50 demonstrated that the IZ-AC peptide was a monomer in solution over a concentration range of 10 μM–1 mM.

Metal ion selectivity of IZ-AC

To analyze the metal-induced assembly and folding of IZ-AC, we tested Co(II), Ni(II), Zn(II), Cd(II), and Hg(II), which are the ligands of metallothioneins. The helicities of the IZ-AC-metal complexes were measured by CD spectral analyses. Figure 2 shows the CD spectra of a mixture of IZ-AC and various metal ions at a ratio of 1:1, at pH 7.6 and 20 °C. Neither Co(II), Ni(II), nor Zn(II) induced the α-helical structure. On the other hand, IZ-AC exhibited the α-helical structure with a minimum at 222 nm in the presence of Cd(II) and Hg(II). In the case of Hg(II), a higher α-helicity was observed at the Hg(II)/IZ-AC ratio of 1/3. These metal ions were confirmed not to oxidize the thiols by the HPLC analysis.

Cd(II) binding to IZ-AC

A coiled coil structure usually has a ratio of $[\theta]_{222}$ to $[\theta]_{208}$ ($[\theta]_{222}/[\theta]_{208}$) of >1 (Graddis et al., 1993; Zhou et al., 1994; Kohn et al., 1995). However, the IZ-AC-Cd(II) complex showed a $[\theta]_{222}/$

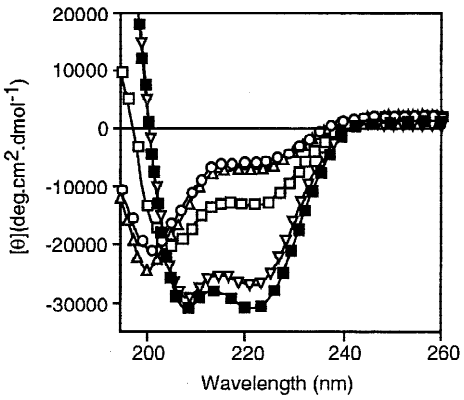

Fig. 2. CD spectra of IZ-AC in the absence and presence of various metal ions at 20 °C and pH 7.6. The concentrations of the peptide and the metal were 20 μM. No metal (circles); Cd(II) (inverted triangles); Zn(II) (triangles); Hg(II) (open squares). The spectra with Co(II) and Ni(II) were almost the same as that of Zn(II) (data not shown). The CD spectrum of a mixture of IZ-AC (20 μM) and Hg(II) (6.7 μM) was shown by closed squares.

$[\theta]_{208}$ ratio of 0.9, suggesting that the IZ-AC-Cd(II) complex has a fluctuation at the end of the coiled coil or an α-helical bundle structure. The α-helicity was increased by the addition of Cd(II) up to a metal/IZ-AC ratio of 1/3 and was followed by a plateau at higher metal concentrations (Fig. 3). The CD spectra had an isosbestic point of 204 nm. The sedimentation equilibrium analyses at the Cd(II)/IZ-AC ratio of 5/1 at pH 7.6 at a peptide concentration range of 20–100 μM gave the molecular mass of 10.6 ± 0.3 K_d (expected mass for trimer was 10.5 K_d), indicating that the IZ-AC peptide was trimeric (Fig. 4). At the higher concentration (500 μM–1 mM), the data were consistent with a trimer model, but nonrandom residuals were also observed, suggesting the tendency the aggregation of the peptide. The trimerization of the peptide was also confirmed by size exclusion chromatography using Sephadex G50. These results, taken together with the NMR result described below, show that the binding of one Cd(II) to the IZ-AC peptide causes it to fold into a triple-stranded α-helical structure. The dissociation constant K_d between IZ-AC and Cd(II) was calculated as 1.5 ± 0.8 μM, determined by curve fitting of a CD titration, as a two state model between the monomer and the trimer (Fig. 3).

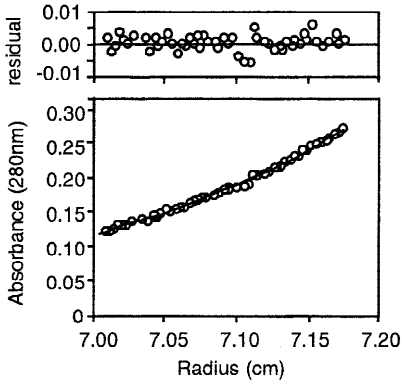

Fig. 4. Representative analytical ultracentrifugation sedimentation data for the IZ-AC/Cd(II) complex. The concentrations of the peptide and the metal were 100 and 500 μM, respectively. The sample was centrifuged at 32,000 rpm at 25 °C. The random distribution of the residuals indicates that the data fit to a single ideal species.

^{113}Cd NMR study of the coordination of IZ-AC to Cd(II)

To identify the Cd(II) coordination state, we measured the ^{113}Cd NMR with a ^{113}Cd(II)/IZ-AC ratio of 1/3. Figure 5 shows the ^{1}H–^{113}Cd HMQC spectrum of $[^{113}Cd](IZ\text{-}AC)_3$ at 27 °C and pH 7.6 with the one-dimensional ^{1}H and ^{113}Cd NMR spectra. A single ^{113}Cd NMR resonance was observed at 572 ppm, which was connected with the two βH protons of the Cys residue at 3.1 and 2.9 ppm. There was no observable ^{113}Cd NMR signal in the other regions. It is empirically known that metal binding sites comprising exclusively O-donor ligands give ^{113}Cd signals in the range of +40 to −180 ppm, sites with one to three N donors give shifts in the range of 40 to ~300 ppm, and sites with S donors give signals with shifts from ~400 to 800 ppm. In the case of plural thiolate ligands coordinated to a ^{113}Cd ion, the ^{113}Cd NMR chemical shift exhibits a downfield shift of 140–200 ppm per thiolate (Summers, 1988). Thus, the ^{113}Cd chemical shift at 572 ppm of $[^{113}Cd](IZ\text{-}AC)_3$ indicates that all three Cys residues of the triple-stranded structure are coordinated to ^{113}Cd(II). This ^{113}Cd chemical shift value coincides excellently with the reported value of a trigonal-planar complex of Cd(II) with three thiolate ligands (577 ppm) (Gruff & Koch, 1989, 1990).

To examine whether a water molecule is coordinated to the ^{113}Cd(II) ion of $[^{113}Cd](IZ\text{-}AC)_3$, the ^{113}Cd NMR spectrum was measured in the presence of 150 mM NaCl. In general, if the external medium is accessible to the metal ion, then the ^{113}Cd NMR signal exhibits downfield shifts with an increase in the chloride ion concentration (Summers, 1988). The ^{113}Cd chemical shift of $[^{113}Cd](IZ\text{-}AC)_3$ was unaffected by the presence of chloride ions (data not shown), suggesting that a water molecule is not coordinated to the metal ion of $[^{113}Cd](IZ\text{-}AC)_3$, leading to a three-coordinate complex. The chloride ion might not be exchangeable with a coordinated water buried inside of the helical bundle. However, the ^{113}Cd-NMR signal of $[^{113}Cd](IZ\text{-}AC)_3$ in 90% H_2O/

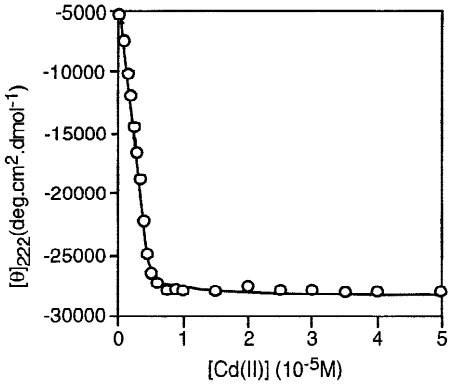

Fig. 3. Cd(II) titration profile of IZ-AC monitored by CD spectroscopy at 20 °C and pH 7.6. The $[\theta]_{222}$ was monitored and plotted as a function of the metal concentration. The peptide concentration was 15 μM. The $[\theta]_{222}$ of the metal-saturated forms of the peptide $[\theta]_s$ was $-28{,}700$ deg cm^2 dmol^{-1}. The data were fitted using a nonlinear least-squares fitting procedure, as described in Materials and methods.

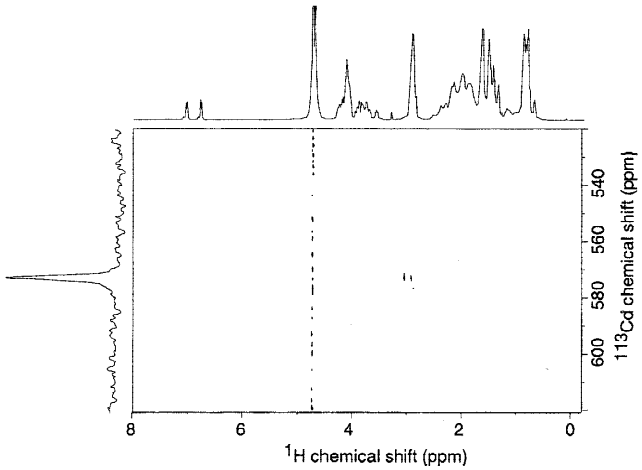

Fig. 5. ^{1}H-^{113}Cd HMQC spectrum of $[^{113}Cd](IZ\text{-}AC)_3$ accompanied with one-dimensional ^{1}H (horizontal) and ^{113}Cd (vertical) NMR spectra at 27 °C, pH 7.6. For the direct ^{113}Cd NMR measurement, the number of scans was 80,000, and the relaxation delay was 2 s. A 100 Hz line broadening was applied.

10% D_2O solution was identical with that in D_2O, whether measured with or without proton decoupling, which also implies no coordination of the water molecule.

Hg(II) binding to IZ-AC

The Hg(II) titration with the peptide showed a rather complicated binding mode. The $[\theta]_{222}$ was monitored as a function of the Hg(II) concentration (Fig. 6). Upon the addition of Hg(II) up to a Hg(II)/IZ-AC of 1/3, the structure of IZ-AC was effectively induced into an α-helical structure. At this point, the α-helicity was maximal at the $[\theta]_{222}$ value of $-35{,}000$, and the IZ-AC-Hg(II) complex showed a CD spectrum exhibiting a coiled coil with a $[\theta]_{222}/[\theta]_{208}$ ratio of 1.06 (Fig. 2). However, the further addition of Hg(II) decreased the α-helicity of the IZ-AC peptide. We measured the UV_{247} absorbance, which is characteristic for a three-coordinate Hg complex (Dieckmann et al., 1997), at various Hg(II)/IZ-AC ratios, while maintaining a constant Hg(II) concentration (Fig. 7). At an IZ-AC/Hg(II) ratio of more than 3, under which IZ-AC formed the coiled coil, the IZ-AC-Hg(II) complex showed UV absorbance at 247 nm with shoulders at 265 and 295 nm. These results indicate that IZ-AC is trimerized, by binding to Hg(II) using three Cys residues, under conditions with an excess of the peptide. However, the UV absorbance at 247 nm decreased when the IZ-AC/Hg(II) ratio fell from 3 to 1, and disappeared at an IZ-AC/Hg(II) ratio of <1 (Fig. 7). This shows that the amount of the three-coordinate Hg(II) decreases and that of the two- or one-coordinate Hg(II) increases as the Hg(II) ratio increases. The size exclusion chromatographic analysis showed that IZ-AC was a trimer as a main product at a Hg(II)/IZ-AC ratio of 1/3. However, the contents of the monomer and dimer of IZ-AC increased with the excess of the Hg(II) concentration (data not shown).

To analyze whether IZ-AC dimer connected by the two-coordinate Hg(II) could form α-helical structure, we prepared the disulfide-linked IZ-AC as a model. The disulfide-linked IZ-AC alone did not exhibit the α-helical structure, even at a concentration of 200 μM (Fig. 8). However, it showed the α-helical structure after the addition of one equivalent of the IZ-AC peptide with a reduced cysteine residue. This result showed that the dimer and monomer of IZ-AC were associated to form the α-helical bundle structure. These results support the above result that IZ-AC formed the α-helical structure when it was trimerized.

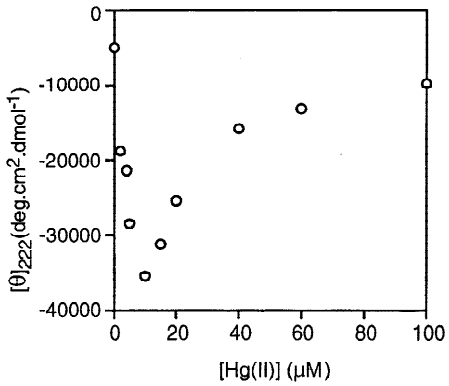

Fig. 6. Hg(II) titration profile of IZ-AC monitored by CD spectroscopy at 20 °C and pH 7.6. The $[\theta]_{222}$ was monitored and plotted as a function of the Hg(II) concentration. The peptide concentration was 30 μM.

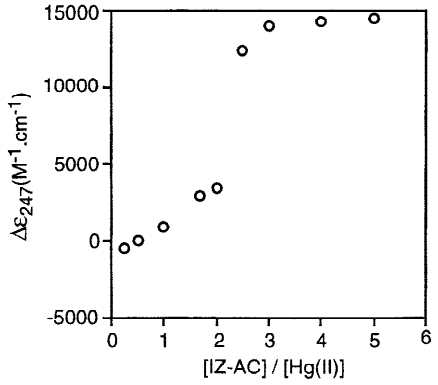

Fig. 7. UV titration of Hg(II) with IZ-AC at pH 7.6. $\Delta\epsilon_{247}(\epsilon_{247\text{IZ-AC+Hg}} - \epsilon_{247\text{IZ-AC}})$ was plotted as a function of the IZ-AC/Hg(II) ratio. The concentration of Hg(II) was kept at 20 μM.

Discussion

We described herein a peptide that undergoes metal ion-induced self-assembly. We placed the metal binding site in the hydrophobic core, because amino acids in the hydrophobic core have more influence on the conformation of the coiled coil structure than those at the solvent exposed sites (Suzuki et al., 1999). We previously described the IZ-3adH peptide, which has two His residues, and binds Ni(II), Co(II), and Zn(II) (Suzuki et al., 1998a). In this paper, we changed the metal ion selectivity by the introduction of Cys residues, which prefer soft bases, instead of His residues. The IZ-AC peptide, without metal ions, was monomeric with a random structure at least up to 1 mM concentration. The IZ-AC peptide did not bind Ni(II), Co(II), or Zn(II), but instead bound Hg(II) and Cd(II), which are "soft" metal ions. The Hg(II) and Cd(II) ions induced the assembly and folding of IZ-AC into the triple stranded α-helical coiled coil.

The binding affinities of various divalent cations to the Cys residue decrease in the order of Hg(II)> Cd(II)> Ni(II)> Zn(II)> Co(II) (Nielson et al., 1985). This order usually parallels those observed in the metallothioneins and the MerR metalloregulatory

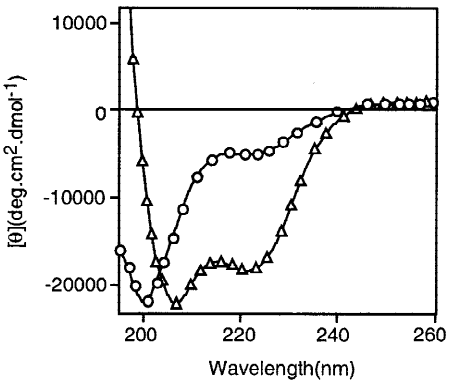

Fig. 8. CD spectral change of the dimerized IZ-AC by the addition of reduced IZ-AC. The CD spectra of the dimerized IZ-AC (20 μM) (circles) and a mixture of dimerized IZ-AC and IZ-AC (20 μM each) (triangles) were measured at 20 °C and pH 7.6.

protein (Nielson et al., 1985; Ralston & O'Halloran, 1990). It was reported that Ni(II) and Zn(II) ions bind metallothioneins with a tetrahedral geometry, and their affinities are much weaker than those of Hg(II) and Cd(II) ions (Nielson et al., 1985; Kägi & Schäffer, 1988; Robbins et al., 1991; Jiang et al., 1994). For example, Cd(II) has a 10,000-fold higher affinity over Zn(II) in metallothioneins (Kägi & Schäffer, 1988). This coincides with the present result that neither Ni(II) nor Zn(II) induced the α-helical structure of IZ-AC, but Cd(II) did (Fig. 2). The binding affinities of Ni(II), Zn(II), and Co(II) may not be sufficient to induce the α-helical structure, while Cd(II) can interact with the three Cys residues to induce the α-helical structure of IZ-AC.

The coordinate number of Cd(II) is usually 4 or 6 (Summers, 1988). It was reported that Cd(II) exhibits tetrahedral geometry in metallothioneins (Kägi & Schäffer, 1988). A trigonal-planar complex of Cd(II) with sterically hindered thiolate ligands was also reported, although it is extremely rare. The complex has a pinwheel conformation with approximately C_{3h} symmetry, and exhibits a ^{113}Cd resonance at 577 ppm (Gruff & Koch, 1989, 1990). The ^{113}Cd-IZ-AC complex has a ^{113}Cd chemical shift of 572 ppm, suggesting the trigonal-planar complex. The steric hindrance and the high symmetry of the α-helical bundle structure may allow $[^{113}Cd](IZ\text{-}AC)_3$ to form a symmetric trigonal-planar complex. The hydrophobic moiety around the metal binding site may also prevent the access of water to the metal ion, by the fact that the chloride ion did not affect the ^{113}Cd chemical shift. These results suggest that Cd(II) forms a trigonal-planar complex with the IZ-AC peptide.

In metallothioneins, Hg(II) can form sulfur complexes with diagonal, trigonal, and tetragonal coordinations, depending on the metal-protein ratios (Ralston & O'Halloran, 1990; Jiang et al., 1994; Fowle & Stillman, 1997). On the other hand, Hg(II) is suggested to be trigonal in the MerR protein (Wright et al., 1990b; Utschig et al., 1995; Zeng et al., 1998). Hg(II) efficiently induced the folding and the α-helical structure of IZ-AC with the trigonal form, judging from the UV absorbance. The first and second bindings of Hg(II) to the sulfhydryl group of a Cys residue are extraordinarily strong as compared with the third binding (Dieckmann et al., 1998; Wright et al., 1990a). Above a Hg(II)/IZ-AC ratio of 0.5, therefore, the Hg(II) should bind to only one or two IZ-AC peptides, and subsequently, the peptide would existed mainly as a monomer and dimer mixture. This is supported by size exclusion chromatographic analysis. The monomer and dimer of the IZ-AC peptide would not assume the α-helical conformation, in contrast to its trimer form. In fact, the disulfide-linked dimer of IZ-AC did not show the α-helical conformation, whereas it displayed the α-helical structure with the addition of one equivalent of IZ-AC. These results indicate that only when Hg(II) has three coordinates interacting with the three Cys residues from three IZ-AC peptides, the IZ-AC was trimerized and formed the α-helical structure. In contrast to the vastly different affinities of Hg(II) between two and three coordinations, the affinity of Cd(II) only slightly decreases from mono to tetracoordination (Ralston & O'Halloran, 1990). Therefore, an excess of Cd(II) did not destabilize the trimerized structure of IZ-AC.

Thus, we have described a metallopeptide, in which one "soft" metal bound in the hydrophobic core induces the peptide to assemble into the triple stranded α-helical structure. In addition to the MerR protein, there are other natural proteins with functions that are regulated by metal ions, such as the yeast ACE1 transcription factor (Casas-Finet et al., 1991), the HIV-2 integrase (Eijkelenboom et al., 1997), and the diphtheria toxin repressor (White et al., 1998). Thus, combining the IZ-AC peptide and a domain that exerts its functions by self di- or trimerization should facilitate the creation of de novo designed proteins with functions controlled by metal ions.

Materials and methods

Peptide synthesis and purification

IZ-AC 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), and Cys(Trt). 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 reversed-phase HPLC on a YMC-Pack ODS-A column (10 mm i.d. × 250 mm, 5 μm, YMC Inc., Kyoto, Japan) with a linear gradient of 20 to 60% CH_3CN/H_2O containing 0.1% TFA over the course of 40 min. The final product was characterized by analytical HPLC and was confirmed by MALDI-TOF mass spectrometry, *m/z*: 3,490 (calcd: 3,490).

Circular dichroism (CD) spectroscopy

CD measurements were performed on a Jasco-720 spectropolarimeter, using a 1 mm cuvette at 20 °C. The peptide concentration was determined by measuring the tyrosine absorbance in 6 M guanidium chloride, using $\epsilon_{275} = 1{,}450\ M^{-1}\,cm^{-1}$ (Padmanabhan & Baldwin, 1991). The mean residue ellipticity $[\theta]$ is given in units of $deg\,cm^2\,dmol^{-1}$. CD spectra were obtained in 20 mM phosphate buffer (pH 7.6) at a peptide concentration of 20 μM in the absence and presence of 20 μM Zn(II), Co(II), Ni(II), Cu(II), Cd(II), Hg(II), and 6.7 μM Hg(II). Metal ion titrations were carried out in the same buffer by monitoring $[\theta]_{222}$ as a function of the metal concentration, which ranged from 1 to 200 μM. The peptide concentration was 15–30 μM. To avoid the probable oxidation of IZ-AC by air, the buffer was purged with nitrogen, and all samples were immediately measured after preparation.

Assuming a two state mode, $1/3M + P \times 1/3P_3M$, the dissociation constant K_d is expressed as $K_d = [P][M]^{1/3}/[P_3M]^{1/3}$, where $[P]$, $[M]$, and $[P_3M]$ are the free peptide, the free metal, and the peptide-metal complex concentrations, respectively. When the total peptide and the total metal concentrations are P_t and M_t, respectively, and f is the folded fraction, then $K_d = (P_t - fP_t)(M_t - fP_t/3)^{1/3}/(fP_t/3)^{1/3}$. The K_d for the metal was determined from a nonlinear least-squares fit, using the KaleidaGraph program (Synergy Software, Reading, Pennsylvania) (Suzuki et al., 1998a).

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, 100, and 1,000 μM in phosphate buffer (20 mM, pH 7.6) containing five equivalent of $CdCl_2$. The samples were independently rotated at 25,000 and 32,000 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 $CdCl_2$, using Origin Sedimentation Single Data Set Analysis (Beckman, Palo Alto, California). A partial specific volume of 0.761 mL/g was calculated for IZ-AC, using the method of Cohn and Edsall (1943).

Size exclusion chromatography

IZ-AC (10 μM and 1 mM), and a mixture of IZ-AC (10 μM–1 mM) and five equivalent of $CdCl_2$, and a mixture of IZ-AC (30 μM) and Hg(II) in the ratio of 6:1, 3:1, and 1:3 were 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 (i.d.) $\times$ 9 cm) and were eluted with the same buffer at pH 7.0. One fraction of 90 μL was collected and monitored at a wavelength of 230 nm. As the peptide standards, GCN4-pLI (Harbury et al., 1993), IZ (Suzuki et al., 1998a), and GCN4-p1 (Harbury et al., 1993) were used for tetramer, trimer, and dimer, respectively.

UV/visible titration

UV/visible spectra were collected at room temperature using a Beckman DU640 spectrophotometer. The blank was x μM IZ-AC in 20 mM phosphate buffer (pH 7.6), and the sample was x μM IZ-AC and 20 μM $HgCl_2$ in 20 mM phosphate buffer (pH 7.6), where x = 5, 10, 20, 30, 40, 50, 60, 80, or 100. $\Delta\epsilon_{247nm}$ ($\epsilon_{247nm\ IZ\text{-}AC+Hg} - \epsilon_{247nm\ IZ\text{-}AC}$) was obtained as A/cl, where c was 2×10^{-5} M, and l was 1 cm.

NMR spectroscopy

^{113}Cd metal (95 atom %) was obtained from ISOTEC Inc. (Miamisburg, Ohio). The $[^{113}Cd]SO_4$ solution was prepared as previously described (Kanaori et al., 1996). The preparation of the $[^{113}Cd](IZ\text{-}AC)_3$ complex was achieved by adding a peptide solution to a $[^{113}Cd]SO_4$ solution and adjusting the pH to 7.6 with 0.5 N NaOD and 0.5 N D_2SO_4 solutions, to avoid the influence of halide ions on the ^{113}Cd chemical shift. The final concentration of the $[^{113}Cd](IZ\text{-}AC)_3$ complex was 1.5 mM. All of the NMR measurements were performed on a Bruker ARX–500 spectrometer at 27 °C with a 5 mm tunable broad-band probe for direct measurement of the ^{113}Cd resonance, and with a 5 mm inverse probe for the ^1H–^{113}Cd HMQC spectroscopy. The ^{113}Cd chemical shift was referred to the resonance position of 0.1 M $Cd(ClO_4)_2$. The ^{113}Cd acquisition parameters were as follows: sweep width of 33,000 Hz, relaxation delay of 2 s, and pulse width of 4 μs (45° pulse) without proton decoupling.

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