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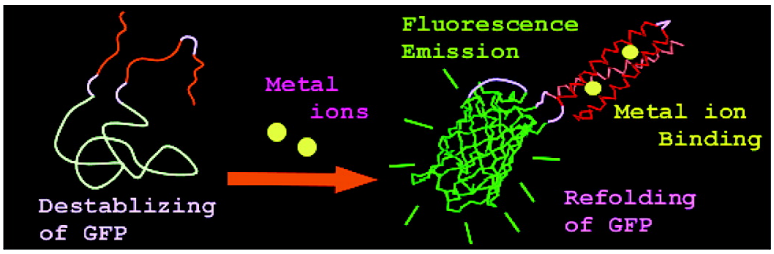

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Metal-ion-dependent GFP Emission in Vivo by Combining a Circularly Permutated Green Fluorescent Protein with an Engineered Metal-Ion-Binding Coiled-coil

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Abstract: Coordination of metal ions significantly contributes to protein structures and functions. Here we constructed a fusion protein, consisting of a de novo designed, metal-ion-binding, trimeric coiled-coil and a circularly permutated green fluorescent protein (cpGFP), where the fluorescent emission from cpGFP was induced by metal ion coordination to the coiled-coil. A circularly permutated GFP, ^{191}cpGFP^{190}, was constructed by connecting the original N- and C-termini of GFP_{UV} by a GGSGG linker and cleaving it between Asp^{190} and Gly^{191}. The metal-ion-binding coiled-coil, IZ-HH, was designed to have three α-helical structures, with 12 His residues in the hydrophobic core of the coiled-coil structure. IZ-HH exhibited an unfolded structure, whereas it formed the trimeric coiled-coil structure in the presence of divalent metal ions, such as Cu^{2+}, Ni^{2+}, or Zn^{2+}. The fusion protein ^{191}cpGFP^{190}-IZ-HH was constructed, in which ^{191}cpGFP^{190} was inserted between the second and third α-helices of IZ-HH. *Escherichia coli* cells, expressing ^{191}cpGFP^{190}-IZ-HH, exhibited strong fluorescence when the Cu^{2+} and Zn^{2+} ions were present in the medium, indicating that they passed through the cell membrane and induced the proper folding of the ^{191}cpGFP^{190} domain. This strategy, in which protein function is regulated by a metal-ion-responsive coiled-coil, should be applicable to the design of various metal-ion-responsive, nonnatural proteins that work both in vitro and in vivo.

Introduction

The de novo design of protein function is an important goal in protein engineering.[1] However, it still remains a significant challenge, even with the current sophisticated computational and random selection-based protein-design techniques. By contrast, the construction of combination proteins, which contain two or more functional domains from natural or nonnatural proteins and/or peptides, provides a method for the versatile design of protein function. For example, the combination of fluorescent proteins with ligand binding or enzyme−substrate domains allows the creation of novel fluorescence-resonance-energy-transfer (FRET)-type sensor proteins, which facilitate the visualization of the location of the ligands or the enzyme activity in vivo.[2] The combination of designed DNA binding domains with transcription activation domains has yielded novel artificial transcription factors.[3] In most cases, especially for use in vivo, the functional domains are mainly limited to those from natural proteins, but the introduction of designed, nonnatural protein domains should expand and develop the versatility of these kinds of combination proteins.[4]

Previously, we have de novo designed various types of metal-ion-binding, coiled-coil peptides. They form the α-helical, trimeric coiled-coil structure only in the presence of the metal ions; otherwise, they assume the random coiled structure.[5,6] The design concept for the metal-ion-binding coiled-coil is the destabilization of the hydrophobic packing by a His or Cys substitution at the hydrophobic core. The hydrophobic packing

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is particularly crucial for the coiled-coil protein to maintain the folded structure,[7] and thus the His or Cys mutants result in the random coiled structure. The His residue has the potential to coordinate medium metal ions, such as Ni^{2+}, Cu^{2+}, and Zn^{2+}, while the Cys residue can coordinate soft metal ions, such as Cd^{2+}, Cu^{+}, and Hg^{2+}. The formation of intermolecular-bridge between the His or Cys residues from different peptide chains promotes the assembly of the peptide chains into the coiled-coil structure. These domains should function as a metal-ion-responsive unit for the design of metal-ion-gated combination proteins, with function that can be manipulated by metal ions.

On the other hand, circular permutation is another method used to mutate the original proteins, by exchanging the position of the N- and C-termini in the folded protein scaffold.[8] This method is utilized to judge the well-folded area of the original protein scaffold, because the placement of flexible peptide chains, instead of the connected chain, usually affects the protein folding and destabilizes the structure. Recently, circular permutation has been used as a tool to temporarily destabilize a protein structure. The circular permutant was then refolded, and the original protein function was restored after the introduction of another interaction that brought the new N- and C-termini close together. This methodology has been used to endow several natural proteins with a switch character.[4c,9]

Here we designed a combination protein including the de novo designed metal-ion-responsive trimeric coiled-coil and the circularly permutated green fluorescent protein (cpGFP). Metal coordination to the coiled-coil domain is expected to affect the folding and fluorescent function of the cpGFP domain. The metal-ion-responsive, trimeric coiled-coil was designed as a single peptide chain, in which the three α-helices are connected with flexible linkers and 12 His residues are located at the hydrophobic sites. This protein is expected to fold into the trimeric coiled-coil structure only in the presence of metal ions, such as Ni^{2+}, Cu^{2+}, or Zn^{2+}. We selected cpGFP, in which the fluorescence emission is restored by the aid of a stable, trimeric coiled-coil introduced at the cleaved sites for the circular permutation. Then, we constructed the combination protein, composed of the metal-ion-responsive trimeric coiled-coil and cpGFP. We examined the fluorescence characteristics of this combination protein in *Escherichia coli* cells.

Materials and Methods

Taq DNA polymerase, restriction enzymes, T4 DNA ligase, and alkaline phosphatase were purchased from Takara Shuzo, Japan. General chemicals, isopropyl β-D-thiogalactopyranoside (IPTG), bacto-tryptone, agar, and yeast extract (Wako Chemicals, Japan), ampicillin (Meiji Seika, Japan), Agarose ME (Iwai Chemicals, Japan), glycogen (Roche Diagnostics, Japan), and reagents were used without further purification.

Expression and Purification of the cpGFP Variant Proteins. The DNA fragments, with *Nco*I and *Hin*dIII restriction enzyme sites at each end, encoding the cpGFP mutants were inserted into the pRSET plasmid vector (Invitrogen). *E. coli* BL21(DE3) cell were transformed with these plasmids and were cultured at 37 °C for 5 h in 250 mL of LB medium and then at 25 °C for 15 h in the presence of 1 μM IPTG. The cells were harvested, resuspended in 7 mL of 20 mM Tris-HCl (pH 7.5) buffer, and sonicated. The suspension was centrifuged, and the supernatant fraction was separated. The supernatant was applied to a His-Bind resin column (Novagen, 5 mL). The resin was washed with 20 mM Tris-HCl (pH 7.9) buffer (30 mL) containing 500 mM NaCl and 5 mM imidazole, and then with the same buffer containing 500 mM NaCl and 60 mM imidazole (50 mL). The target protein was eluted with the same buffer containing 1 M imidazole (20 mL), and each fraction was analyzed by sodium dodecyl sulfate−15% polyacrylamide gel electrophoresis (SDS−PAGE). The fractions containing the target protein were collected, and this solution was dialyzed five times against 2 L of 20 mM phosphate buffer (pH 7.5) at 4 °C. The concentration of each protein solution was determined by the A_{280} values in a 6 M guanidine hydrochloride solution, by use of the ϵ_{280} value (31 400 $M^{-1}\cdot cm^{-1}$) of the cpGFP mutants.

Expression and Purification of IZ-H and IZ-HH Proteins. The DNA fragments, with *Nco*I and *Hin*dIII restriction enzyme sites at each end, encoding IZ-H and IZ-HH were inserted into the pET-32a(+) plasmid vector (Novagen). *E. coli* BL21(DE3) cells, transformed with these plasmids, were cultured at 37 °C for 5 h in 250 mL of LB medium and then at 37 °C for 3 h in the presence of 1 mM IPTG. Disruption of the cells and suspension of the expressed protein by the His-Bind resin (Novagen) were carried out by the same method described above. The fractions containing the target protein were collected and dialyzed three times against 2 L of 20 mM Tris-HCl buffer (pH 8.4) containing 150 mM NaCl and 2.5 mM $CaCl_2$, at 4 °C. The dialyzed solution was subjected to thrombin digestion, to remove the thioredoxin fusion protein from the IZ-H and IZ-HH proteins. Finally, the mixtures were subjected to reverse-phase HPLC separation, with a linear gradient of MeCN/H_2O containing 0.1% TFA as the eluent. The purity of the IZ-H and IZ-HH proteins was confirmed by SDS−15% PAGE.

Circular Dichroism Measurement. All CD measurements were carried out on a Jasco J-820 spectrometer, with a 2 mm or 1 cm path-length cuvette. CD spectra of IZ-H and IZ-HH (0.1 or 5 μM) were measured in 10 mM phosphate buffer (pH 6.8) containing 100 mM NaCl, in the presence (0.2 or 10 μM) or absence of Ni^{2+}, Cu^{2+}, or Zn^{2+}. The mean residue ellipticity, [θ], is given in units of degrees·centimeter^2·decimole^{-1}.

Isothermal Titration Calorimetry Measurement. ITC experiments were carried out on a MicroCal MCS calorimeter interfaced with a microcomputer. All samples were in 10 mM phosphate buffer (pH 6.8) containing 100 mM NaCl, and all solutions were thoroughly degassed by use of the degassing equipment provided with the instrument. The metal ion solution was titrated into the IZ-HH solution. Each titration consisted of a preliminary 2-μL injection followed by 23 subsequent 5-μL additions, which were performed over 10-s periods at 240-s intervals. The heat for each injection was subtracted from the dilution heat of the titrant, and each corrected heat was divided by the moles of metal ion injected. Data were analyzed with the Origin software supplied by MicroCal.

Fluorescence Measurement of cpGFP Variants. The fluorescence measurements were performed with a Hitachi F-4500 fluorescence spectrophotometer, with a 1 cm path-length cuvette. The emission spectra between 480 and 600 nm were measured, with excitation at 330 nm. The measurements were performed in 20 mM sodium phosphate (pH 7.5) at room temperature. The protein concentration was 0.1 μM.

Observation of Fluorescence Emission of *E. coli* Cells Harboring the ^{191}cpGFP^{190}-IZ-HH Gene. *E. coli* BL21(DE3) cells, transformed with the plasmid vector containing the ^{191}cpGFP^{190}-IZ-HH gene, were cultured at 37 °C for 5 h in 3 mL of LB medium, and then 1 μM IPTG and 500 μM of $CuCl_2$, $ZnCl_2$, or $NiCl_2$ were added. The incubation was carried out at 25 °C for 15 h. The *E. coli* cells were harvested, resuspended in 20 mM phosphate buffer (pH 7.5), and monitored for fluorescence emission under UV irradiation.

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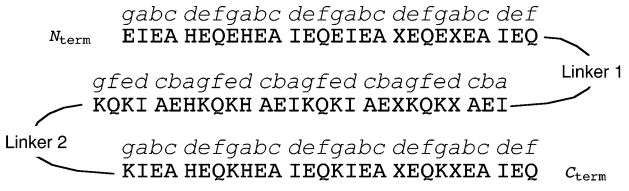

Linker1: GSAGGSGGGGSGGGGSSGGG

Linker2: GGSGGGGSGGGGSGGGGSSG
for **IZ-H**(X=I), or **IZ-HH**(X=H)

GSAG^{191}**cpGFP**^{190}GSSGGG
for ^{191}**cpGFP**^{190}**-IZ-H** or ^{191}**cpGFP**^{190}**-IZ-HH**

Figure 1. Amino acid sequences of IZ-H, IZ-HH, ^{191}cpGFP^{190}-IZ-H, and ^{191}cpGFP^{190}-IZ-HH. The positions of the coiled-coil heptad are indicated by italicized characters above the amino acid sequences. Vertically aligned groups of three amino acids are located in the same plane, perpendicular to the superhelical axis of the coiled-coil structure.

Results and Discussion

Design of a Metal-ion-responsive, Trimeric Coiled-coil Protein. The α-helical coiled-coil has a representative amino acid sequence of $(defgabc)_n$ heptad repeats and forms a superhelix with 2−5 α-helices. The a- and d-positions are usually occupied by hydrophobic residues and form a hydrophobic core.[10] Previously, we de novo designed IZ, [YGG-$(IEKKIEA)_4$] (defgabc),[11] which forms the trimeric coiled-coil structure after the assembly of the three peptide chains. Then, on the basis of IZ, we created various types of triple-stranded α-helical coiled-coils, by fine-tuning the hydrophobic core interactions among each chain. For example, AAB- and ABC-type heterotrimeric coiled-coils,[12] metal-ion-responsive coiled-coils,[5,6] and pH-responsive coiled-coils[13] were successfully designed and constructed. In the case of the metal-ion-responsive coiled-coil, IZ-3adH, destabilizing the hydrophobic core packing, by replacing two sets of Ile-Ile-Ile with His-His-His at the d- and a-positions of the third heptad of the three peptide chains, impaired the coiled-coil structure.[5] On the other hand, the metal coordination among these His residues enabled formation of the trimeric, parallel coiled-coil structure. Here we constructed a metal-ion-responsive coiled-coil protein, by connecting three antiparallel α-helices, with two or four His mutations at the d- and a-positions, with two flexible linkers. To increase the effect of the metal ion and decrease that of linker portions on the coiled-coil formation, we designed the Gly-rich sequences of 20 residues as the linkers.

The sequences of the designed coiled-coil proteins (IZ-H and IZ-HH) are illustrated in Figure 1. In order to arrange the three α-helices into the antiparallel orientation, both the e- and g-positions of the first coiled-coil-forming peptide are occupied by Glu, and those of the second one are Lys, while the e- and g-positions of the third one are Glu and Lys, respectively. These amino acid residues are expected to form salt-bridge interactions at the interface of adjacent strands after antiparallel, trimeric coiled-coil formation. IZ-H has a total of six His substitutions at the successive a- and d-positions on one side of the α-helices. On the other hand, IZ-HH has 12 His substitutions on both sides of the α-helices, to increase the effect of metal ion binding on the coiled-coil formation. IZ-H is expected to incorporate one divalent metal ion, whereas IZ-HH should have two divalent metal ions in the hydrophobic core. The Gln mutation at the f-positions destabilized the trimeric coiled-coil structure of IZ.[11] In order to further destabilize the coiled-coil formation of IZ-H and IZ-HH in the absence of metal ions, the Gln mutations at the f-positions were used in the design of the amino acid sequences.

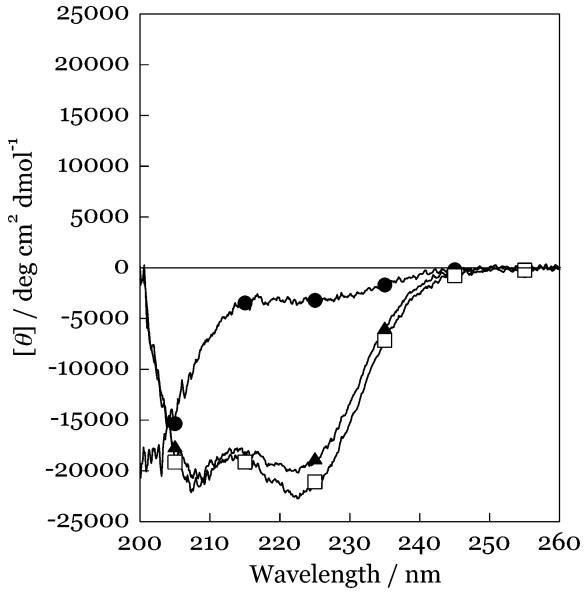

Figure 2. CD spectra of IZ-H without metal ions (▲) and IZ-HH without metal ions (●) or with Cu^{2+} (□). Measurements were performed with protein (5 μM) and metal ions (10 μM) in 20 mM phosphate buffer (pH 6.8).

The IZ-H and IZ-HH proteins were produced with the *E. coli* expression system. To analyze their structures, their far-UV CD spectra were observed (Figure 2). IZ-H shows minima at 208 and 222 nm in the CD spectrum, indicating the formation of helical bundle structure or partially unfolded coiled-coil even in the absence of Cu^{2+}. When we previously designed the metal-ion-binding, α-helical coiled-coil peptide IZ-3adH,[5] it showed a clear metal-ion-dependent structural transition. Hence, connecting the three α-helical peptides with the linkers strengthened the interactions between them, which promoted folding into the coiled-coil structure, despite the hydrophobic packing destabilization by the His mutations. The urea denaturation experiments revealed that IZ-H maintained the fully folded structure, even at a 6 M urea concentration (data not shown).

By contrast, in the absence of Cu^{2+}, the CD spectrum of IZ-HH (5 μM) did not show any significant spectral peaks from 200 to 260 nm but showed a minimum less than 200 nm, indicating that it adopts a random coil structure. However, obvious spectral peaks at 208 and 222 nm, characteristic of an α-helical structure, were observed after the addition of 2 equiv of divalent metal ions (10 μM), such as Cu^{2+}, Ni^{2+}, or Zn^{2+}. The ratio of the $[\theta]$ values at 208 and 222 nm was 1.02, indicating the coiled-coil structure.[14] As the subsequent addition of metal ions did not generate any other effects on the shape of its CD spectrum, the binding of these metal ions for IZ-HH is saturated with only 2 equiv of the metal ions (data not shown).

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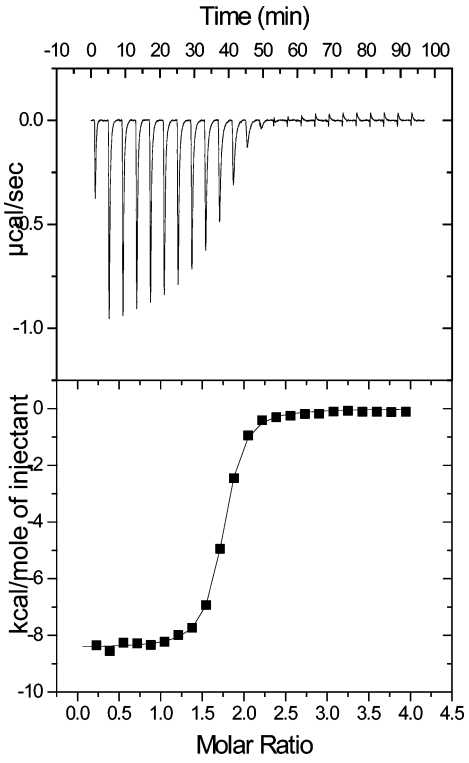

Figure 3. Isothermal titration calorimetry measurements in complex of IZ-HH with Ni^{2+}: [IZ-HH] = 15 μM, [Ni^{2+}] = 0−52.5 μM, 10 mM phosphate (pH 6.8), 100 mM NaCl, 30 °C.

Table 1. K_d Values of IZ-HH for Metal Ions

metal ion	Ratio	ΔH (cal mol^{-1})	ΔS (cal mol^{-1} K^{-1})	K_d (nM)
Ni^{2+}	1.7	−8400	3.7	130
Cu^{2+}	1.9	−14 300	−14	60.0
Zn^{2+}	1.7	−3300	17	570

In order to determine the dissociation constants, K_d, by IZ-HH for each metal ion, we carried out titration experiments by measuring the far-UV CD spectra with a lower concentration of IZ-HH (0.1 μM). However, even 2 equiv of divalent metal ions (0.2 μM) still induces a fully folded structure (data not shown). Hence, we carried out the isothermal titration calorimetry (ITC) experiments to determine the dissociation constants, K_d, of IZ-HH and each metal ion. The exothermic energy fluxes were observed for each titration experiment (Figure 3). Curve-fitting analyses determined each thermodynamic parameter summarized in Table 1. The stoichiometry of metal/IZ-HH complexes was evaluated to be approximately 2, indicating that two metal ions bound to one IZ-HH. The K_ds for first and second metal binding to IZ-HH were considered to be almost identical from the curve-fit analyses. The order of binding affinity is Ni^{2+} (130 nM), Cu^{2+} (60.0 nM), and Zn^{2+} (570 nM). This order has a similar tendency as IZ-3adH. We concluded that IZ-HH is a suitable candidate as a metal-ion-responsive, trimeric coiled-coil protein, which could possibly be combined with cpGFP, to allow the fluorescence emission to be manipulated by metal ion coordination.

Design of Circularly Permutated GFP. Green fluorescent protein (GFP) is composed of 238 amino acid residues and forms

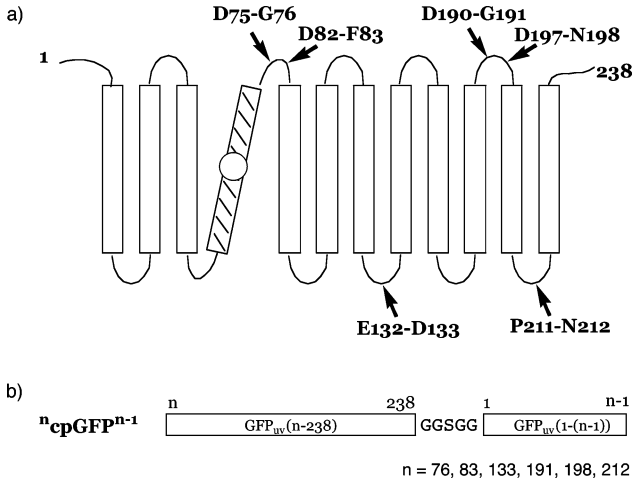

Figure 4. (a) Structure of GFP. Open and hatched rectangles indicate the 11 β-strands and the one α-helix, respectively. The circle indicates the position of the Ser^{65}-Tyr^{66}-Gly^{67} triad, which forms the fluorescent chromophore. The positions cleaved for the circular permutation are indicated by arrows. (b) Schematic illustration of the examined cpGFPs (^{n}cpGFP^{n-1}; n = 76, 83, 133, 191, 198, and 212). The original N-− and C-termini were connected with a GGSGG linker.

a β-barrel structure, containing 11 β-strands and one α-helix (Figure 4a).[15] Following the proper folding of the peptide chain, spontaneous oxidation of three successive amino acids (Ser^{65}, Tyr^{66}, and Gly^{67})[16] yields the fluorescent chromophore, and then the protein exhibits fluorescence. Due to the ease of genetically introducing the fluorescent label to various natural proteins, GFP is currently quite popular in the cell biology field. Several cpGFPs were previously constructed, not only to examine the folding and stability of the protein structure or the oxidation mechanism of GFP but also to construct GFP-based sensor proteins.[9,17] Topell et al.[18] constructed many cpGFPs of wtGFP, and they reported that the divisions at the 25, 39, 50, 103, 117, 145, 158, 174, and 229 positions still retained fluorescence, whereas those at the 10 and 131 positions did not exhibit fluorescence.

In order to construct new cpGFPs, which exhibit fluorescence only when combined with the trimeric coiled-coil, we first selected a cpGFP that did not exhibit fluorescence as follows. We prepared plasmids containing the genes of cpGFPs, divided at the loop regions at the 76, 83, 133, 191, 198, and 212 positions of GFP, and we connected the original N- and C-termini with a GGSGG linker. We named these cpGFP variants ^{76}cpGFP^{75}, ^{83}cpGFP^{82}, ^{133}cpGFP^{132}, ^{191}cpGFP^{190}, ^{198}cpGFP^{197}, and ^{212}cpGFP^{211}, respectively (Figure 4b). *E. coli* BL21(DE3) cells were transformed with these plasmids and incubated on culture plates at 37 °C for 15 h. The colonies of *E. coli* cells were analyzed to determine whether they exhibited fluorescence under UV light. The colonies harboring the

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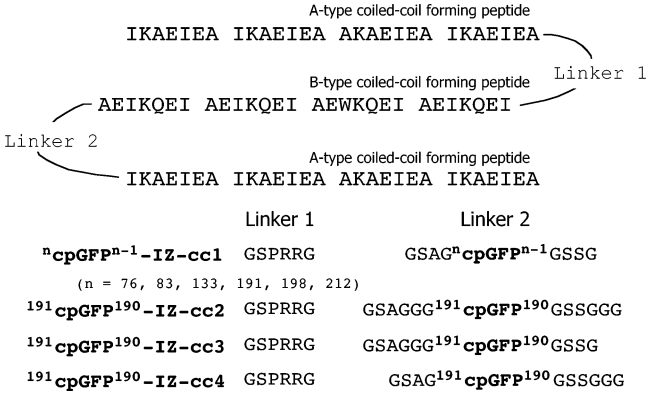

Figure 5. Structure of ^{191}cpGFP^{190}- AAB-type coiled-coil peptide fusion proteins. Each protein has different linker lengths between the ^{191}cpGFP^{190} and coiled-coil-forming peptides.

Table 2. Comparison of Linker Length with Fluorescence Intensities of the Combination Proteins

GFP variants	fluorescence intensity[a]
GFP_{UV}	100
^{191}cpGFP^{190}	<2
^{191}cpGFP^{190}-cc1	4
^{191}cpGFP^{190}-cc2	23
^{191}cpGFP^{190}-cc3	7
^{191}cpGFP^{190}-cc4	55

[a] All of the fluorescence was observed at a protein concentration of 0.1 μM. The intensity values described are compared to that of GFP_{UV}, and they include 10% experimental errors.

aforementioned cpGFP genes did not show any fluorescence. Therefore, we used these for further examinations.

When we combined the six cpGFPs selected above with a trimeric coiled-coil, we first used the stable AAB-type heterotrimeric coiled-coil to construct the ^{191}cpGFP^{190}-IZ-cc1 mutants (Figure 5).[10] The three α-helical peptides of the AAB-type coiled-coil have an antiparallel orientation, and the first and second α-helices are connected with GSPRRG (linker 1). In the linker 2 domain, the cpGFP variants were inserted via tetrapeptide linkers, GSAG for the N-terminal side and GSSG for the C-terminal side of the cpGFPs. The ^{191}cpGFP^{190}-IZ-cc1 mutants were analyzed to determine whether they restored their fluorescence after connecting with the coiled-coil protein. On the plate assay, the *E. coli* colony harboring the combination protein genes encoding ^{191}cpGFP^{190}-IZ-cc1 showed detectable fluorescence. However, the other five combination proteins did not show any fluorescence. Thus, we used ^{191}cpGFP^{190} as the cpGFP domain for the construction of the combination protein. However, the fluorescence intensity was not strong as compared to that of GFP_{UV} (Table 2). Then, we examined the effect of the linker length between the ^{191}cpGFP^{190} and the AAB-type coiled coil protein on the fluorescence intensity. We examined three more sets of ^{191}cpGFP^{190}/AAB-type coiled-coil protein conjugates (^{191}cpGFP^{190}-cc2, -3, and -4). The linker of ^{191}cpGFP^{190} -cc2 is GSAGGG for the N-terminal side and GSSGGG for the C-terminal side of ^{191}cpGFP^{190}. Likewise, ^{191}cpGFP^{190}-cc3 has GSAGGG and GSSG, respectively, while ^{191}cpGFP^{190}-cc4 has GSAG and GSSGGG, respectively (Figure 5). To facilitate the purification of the combination proteins by Ni affinity chromatography, a His tag sequence was incorporated at the N-terminal side of the protein. Unlike the native GFP_{UV}, ^{191}cpGFP^{190} and ^{191}cpGFP^{190}-cc1 were mainly found in the insoluble fraction, and small amounts were purified from the

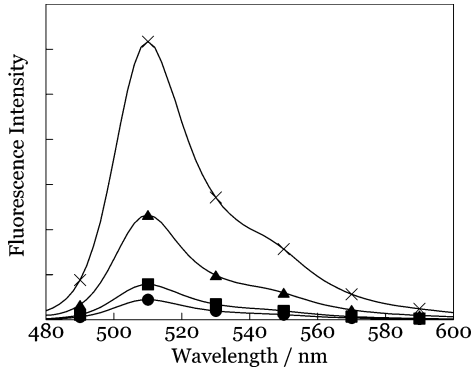

Figure 6. Fluorescence spectra of ^{191}cpGFP^{190}-cc1 (●), ^{191}cpGFP^{190}-cc2 (▲), ^{191}cpGFP^{190} -cc3 (■), and ^{191}cpGFP^{190}-cc4 (×). The fluorescence spectra were measured at a protein concentration of 0.1 μM in 20 mM phosphate (pH 7.5), at room temperature.

soluble fraction. By contrast, ^{191}cpGFP^{190}-cc2 and -cc4 were soluble in the supernatant fraction and easily purified on a Ni affinity column. The fluorescence intensity results for the isolated proteins from each variant are summarized in Figure 6 and Table 2. The fluorescence intensities of ^{191}cpGFP^{190}, ^{191}cpGFP^{190}-cc1, and ^{191}cpGFP^{190}-cc3 were less than 7% that of GFP_{UV} (Table 2). On the other hand, ^{191}cpGFP^{190}-cc2 increased the fluorescence intensity to about a quarter that of GFP_{UV}. The ^{191}cpGFP^{190}-cc4 variant showed even better fluorescence intensity, which was more than half that of GFP_{UV}. Thus, the linker length greatly affects the folding of the ^{191}cpGFP^{190} domain, and better folding increased the fluorescence intensity and the amounts of the protein in the soluble fraction. These results indicate that the combination with the coiled-coil protein induced the proper folding of the ^{191}cpGFP^{190} moiety, and folding ratios were proportional to the solubility and the fluorescence intensity. The ^{191}cpGFP^{190}-cc4 variant was easily detected by its fluorescence on the plate colony assay.

Metal-ion-dependent GFP Emission in Vivo. For construction of the metal-ion-responsive cpGFP, we inserted ^{191}cpGFP^{190}, instead of the second linker of IZ-HH, via the optimized linkers used in ^{191}cpGFP^{190}-cc4, and the mutant protein was named ^{191}cpGFP^{190}-IZ-HH (Figure 1). We also prepared ^{191}cpGFP^{190}-IZ-H for comparison. This combination protein is expected to exhibit the fluorescence, irrespective of the absence or presence of metal ions. When *E. coli* cells transformed with the plasmid containing the genes for ^{191}cpGFP^{190}-IZ-H and ^{191}cpGFP^{190}-IZ-HH were cultured in LB medium, those containing the former gene clearly exhibited the fluorescence, while those harboring the latter gene did not show any fluorescence in the absence of metal ions. However, upon the simultaneous addition of IPTG and Cu^{2+} or Zn^{2+} ions to the culture medium, the *E. coli* cells containing ^{191}cpGFP^{190}-IZ-HH gene clearly showed the fluorescence emission (Figure 7a), as we expected. The fluorescence was increased in a Cu^{2+} concentration-dependent manner (Figure 7b). When we decreased the concentration of metal ions in the culture medium, the fluorescence emissions were detected at 5 μM for Cu^{2+} ion and 100 μM for Zn^{2+}. On the other hand, increasing the concentration of each metal ion strengthened the fluorescence emission, but it did not show saturation until 500 μM of each metal ion.

Interestingly, the addition of Ni^{2+} in the culture medium did not induce detectable fluorescence. IZ-HH has metal-ion-

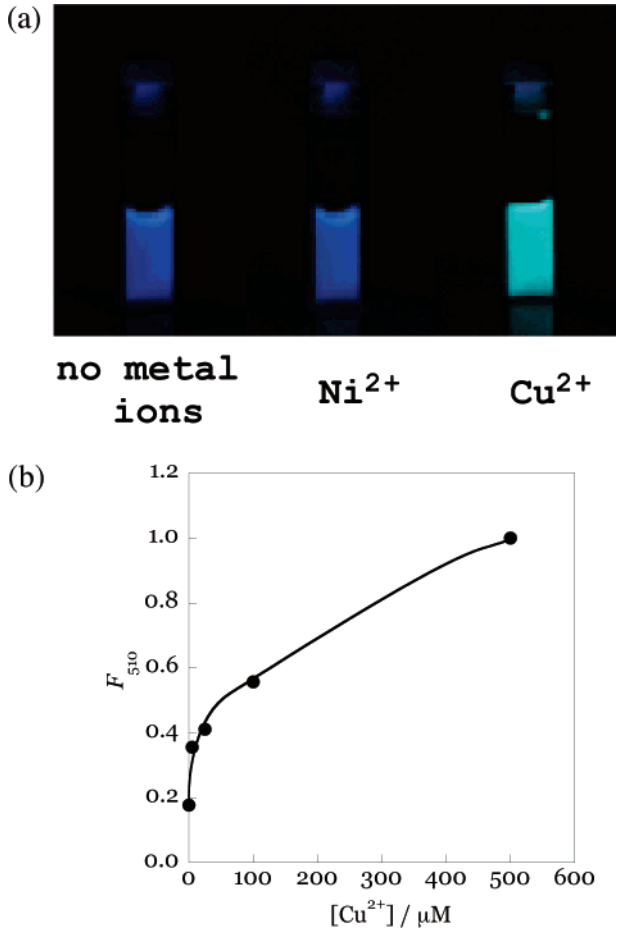

Figure 7. (a) Fluorescence emissions of suspended *E. coli* cells harboring the ^{191}cpGFP^{190}-IZ-HH gene. Protein expression was induced by addition of IPTG (1 μM) in the LB medium (3 mL) without metal ion (left), with 500 μM Ni^{2+} (middle), and with 500 μM Cu^{2+} (right). (b) Copper(II)-dependent fluorescence emission of ^{191}cpGFP^{190}-IZ-HH in *E. coli*. The fluorescence intensities at 510 nm for each Cu^{2+} concentration (0, 5, 25, 100, and 500 μM) were normalized to that for 500 μM Cu^{2+}.

coordination properties similar to those of IZ-3adH, and we showed that IZ-HH bound Ni^{2+} and formed the coiled-coil structure (Figure 2). In our previous work on IZ-3adH, we found that its affinities for $Cu^{2+}$and Ni^{2+} were 10 times higher than that of Zn^{2+}. The weak fluorescence of ^{191}cpGFP^{190} with Zn^{2+} seems to reflect the binding affinity. However, the presence of Ni^{2+} ions in the medium did not induce the fluorescence of cpGFP, in contrast to the case of Cu^{2+} ion (Figure 6). Thus, the ability to restore the fluorescence function of each metal ion is not consistent with the affinity of each metal ion for IZ-3adH. A likely explanation for this might be the difference in the membrane permeability or the metabolism of each metal ion. The NikR protein is expressed in *E. coli* and regulates the expression level of the Ni^{2+} transporter protein.[19] Thus, the incorporation of Ni^{2+} into the cell is strictly regulated, and very little Ni^{2+} passes through the cell membrane from the medium. We presently cannot provide a detailed discussion of the mechanism of metal ion metabolism. However, the metal-ion-dependent fluorescent character of ^{191}cpGFP^{190}-IZ-HH should have potent applicability for monitoring divalent metal ions in living organisms[20] by fluorescent emission.

Conclusions

Combination of the circularly permutated GFP with the de novo designed metal-ion-binding, trimeric coiled-coil protein successfully led to the construction of a metal-ion-responsive GFP. The fluorescence emission was also detected when the combination protein was expressed in *E. coli*, indicating that the protein worked in vivo. Thus, the protein is useful to monitor transition metal ions. This strategy should be applicable to endow various natural proteins with metal-ion-dependent characteristics, leading to the design of metal-ion-dependent, non-natural protein functions.

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