

Lanthanide Ion-Induced Folding of De Novo Designed Coiled-Coil Polypeptides

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To construct a novel isoleucine zipper polypeptide that undergoes a random to coiled coil transition upon lanthanide ion binding, we designed a 37-residue polypeptide (Pep3) with a γ-carboxy glutamic acid (γ-Gla) as metal binding site. Pep3 was designed based on the sequence of the isoleucine zipper polypeptide (Pep1), forming a triple-stranded coiled coil. The coiled-coil structure was destabilized by ionic repulsions among γ-Gla residues at position 21 (hydrophobic 3a position) of one strand. On the other hand, a stable coiled coil formed in the presence of Eu^{3+} ions. This structural change in the designed polypeptide was selective toward Eu^{3+} ion from the circular dichroism (CD) measurements. Furthermore, we designed a lanthanide ion-induced heterotrimeric coiled-coil-assembled system by using two kinds of polypeptides, Pep4 and Pep5. CD spectroscopy, sedimentation equilibrium centrifugation, gel filtration, and HPLC analyses indicate that Pep4 and Pep5 could noncovalently assemble in a 1:2 ratio in the presence of Eu^{3+} ions.

A coiled coil is a superhelix motif that is often used to control protein oligomerization. It is found in many types of proteins, including cytoskeletal and contractile systems,[1,2] transcription factors,[3] viral envelope peptides,[4–6] SNARE complexes,[7] and certain tRNA synthetases.[8] A coiled coil consists of two or more amphipathic α-helices that wrap each other in a left-handed super helix. In general, the hallmark of the coiled coil is a seven residue heptad repeat, $(abcdefg)_n$, with hydrophobic residues at the buried a and d positions and charged residues frequently at the e and g positions.[9,10] The residues at the e and g positions contain oppositely charged residues to form stable inter-helices salt bridges.[11,12]

There are several coiled-coil domains that regulate the functions of natural proteins via large conformational change in response to environmental change. The trimeric protein hemagglutinin of the influenza exhibits pH-dependent conformational change and brings about membrane fusion.[4] In the heat shock transcription factor, the structural change from a random to a coiled-coil structure in response to temperature regulates the expression of the heat shock elements.[13]

Among various external stimuli, metal binding is one of the most attractive targets in constructing de novo designed polypeptides, because metal ions serve a number of important roles in protein structural determinants and catalytic centers in proteins.[14–17] There are several examples of a polypeptide with bidentate ligands self-assembled into a coiled-coil structure in the presence of a transition-metal ion,[18–20] and a lanthanide ion such as Ru^{2+} ion.[21] In many of the triple-stranded coiled-coil polypeptides described previously,[10] metal binding sites have been engineered in a hydrophobic core of the polypeptide. Within the hydrophobic core of de novo designed polypeptides, His residues work as binding sites for transition-metal ions,[22,23] and Cys residues contribute binding sites to heavy metal ions.[24,25] However, lanthanide ion binding sites have not been constructed except one designed double-stranded coiled-coil polypeptide which undergoes a conformational change from a random to a coiled-coil structure upon binding a lanthanide ion to the solvent-exposed γ-carboxyglutamic acid (γ-Gla).[26]

The design of lanthanide complexes with defined architecture is important in view of their applications in biomedical analyses, fluorescence imaging, and cancer phototherapy. Preorganized receptors that encapsulate the luminescent Eu^{3+}, Ce^{3+}, and Tb^{3+} ions to protect their excited states against deexcitation through solvent interaction are also used in other biomedical applications as contrast agents for magnetic resonance imaging, and as catalysts in RNA hydrolysis and in cancer radiotherapy.

Results and Discussion

We have previously constructed a triple-stranded coiled-coil polypeptide (Pep1) and have also designed another polypeptide, Pep2, which undergoes transition-metal-induced self-assembly into a triple-stranded coiled coil (Fig. 1).[27] In this article, we designed a novel lanthanide ion-induced coiled coil formation system. In order to construct this assembled system, Ala and γ-Gla residues were substituted into two Ile

	1	2	3	4	
	efgabcd	efgabcd	efg a bcd	efgabcd	efg
Pep1	YGG EEKIAAI	EKKIAAI	EEK I AAI	EKKIAAI	EEK GGY
Pep2	YGG EEKIAAI	EKKIAA**H**	EEK **H** AAI	EKKIAAI	EEK GGY
Pep3	YGG EEKIAAI	EKKIAA**A**	EEK(Gla)AAI	EKKIAAI	EEK GGY
Pep4	YGG EEKIAAI	EKK**W**AAA	EEK(Gla)AAI	EKKIAAI	EEK GGY
Pep5	YGG EEKIAAI	EKK**A**AAA	EEK(Gla)AAI	EKKIAAI	EEK GGY

Fig. 1. Amino acid sequences of isoleucine zipper polypeptide (Pep1), transition metal ion induced polypeptide (Pep2), and novel designed lanthanide ion induced polypeptides (Pep3, 4, and 5).

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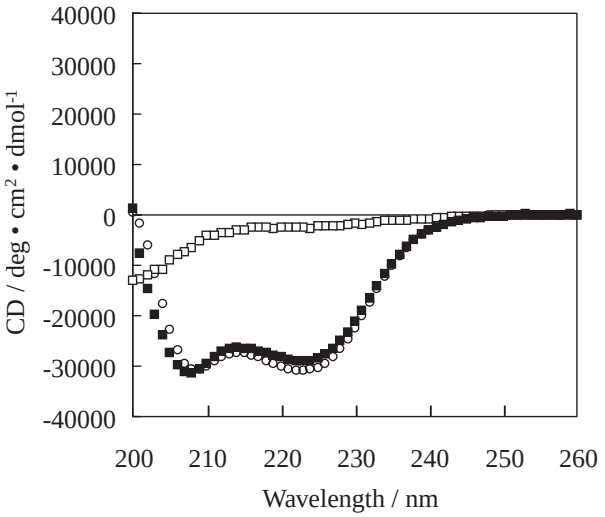

Fig. 2. Circular dichroism spectra of Pep1 (○) and Pep2 in the absence (□) and presence (■) of 40 μM of $NiCl_2$. The measurements were performed in 10 mM Tris-HCl buffer containing 0.1 M NaCl (pH 7.0) at 20 °C. The polypeptide concentrations were 40 μM.

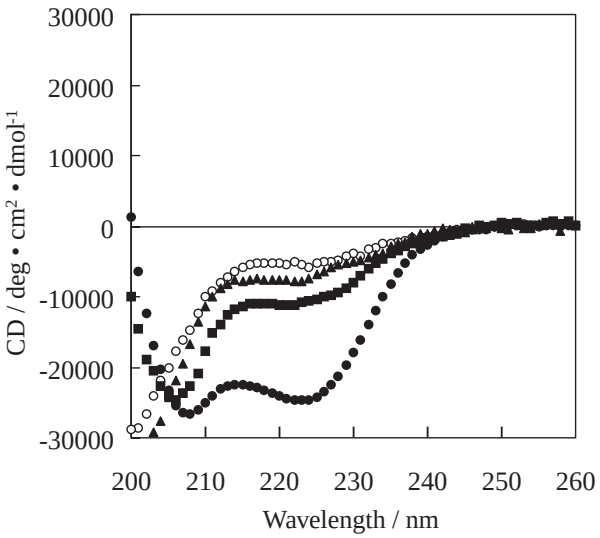

Fig. 3. Circular dichroism spectra of Pep3 in the absence and presence of 40 μM of various metal ions. The measurements were performed in 10 mM Tris-HCl buffer containing 0.1 M NaCl (pH 7.0) at 20 °C. The polypeptide concentrations were 40 μM. No metal (○); Ni^{2+} (▲); Ca^{2+} (■); Eu^{3+} (●). The spectra with Co^{2+}, Zn^{2+}, and Mg^{2+} were almost the same as that of Ni^{2+} (data not shown).

residues in the 2d and 3a positions of the Pep1, respectively. The hydrophilic γ-Gla residue and the small side chain of the Ala residue in the hydrophobic core greatly destabilizes coiled-coil formation and favors a random structure. However, a lanthanide ion is expected to bind to side chain of three γ-Gla residues and compensate for the hole in the hydrophobic core created by three Ala residues from the three novel polypeptide (Pep3) strands. The 3:1 stoichiometry of Pep3/lanthanide ion was supported by CPK modeling around the metal binding site.

The CD spectra of the Pep1 and Pep2 in a Tris-HCl buffer (pH 7.0) are shown in Fig. 2. The spectrum of Pep1 was characteristic for α-helical structure with CD minima at 208 and 222 nm. In contrast, the Pep2 exhibited a minimum at 200 nm, which is characteristic for a random coil structure. In the aqueous buffer solution, the His residues at the hydrophobic core destabilize coiled-coil formation. Addition of Ni^{2+} ions to the Pep2 solution increased the θ_{222} value. These results are the same as those in a previous report.[27]

In this work, we designed new lanthanide-binding coiled-coil polypeptides based on the Pep1. The CD spectra of the lanthanide-binding polypeptide Pep3 are shown in Fig. 3. Pep3 also exhibited the characteristic CD pattern for a random coil structure without any metal ions. This fact indicates that the substitution of the hydrophilic γ-Gla residue and the small side chain of Ala residue for Ile at the hydrophobic core destabilize the coiled-coil structure. To analyze the lanthanide ion-induced folding of the Pep3, we used Eu^{3+} ions. In the presence of Eu^{3+} ions, the spectrum of Pep3 became typical of an α-helical structure. Furthermore, similar CD spectral change was observed when other lanthanide ions such as Tb^{3+} and Ce^{3+}, were added to the Pep3 solution. On the other hand, a significant change in the CD spectra was not observed in the presence of transition metals. Basically, the bidentate carboxylate group in γ-Gla acts as a good ligand for the lanthanide ions.[26] Therefore, lanthanide-complexed Pep3 may form stable coiled-coil structure in the aqueous buffer solution.

We also designed another lanthanide-induced coiled-coil model to use as a fluorescence sensing tool. In order to achieve the fluorescence sensing of lanthanide ion, we added a Trp residue as fluorescence sensitizer near the lanthanide-binding hydrophobic core. In this paper, we used a previously designed AAB-type heterotrimeric coiled coil by the substitution of one amino acid at a hydrophobic position[28] so that we can fix a Trp residue at the hydrophobic core. On the basis of the framework mentioned above, two variants, where the Ile residue at 2a position of Pep3 was replaced with either a Trp (Pep4) or Ala (Pep5) residues (Fig. 1).

The CD spectra of Pep4, Pep5, and a 1:2 mixture of Pep4/Pep5 are shown in Fig. 4. In the presence of Eu^{3+} ions, a 1:2 mixture of Pep4/Pep5 exhibited high helicity and cooperative thermal denaturation profile expected of coiled-coil structure. The CD signal for Pep4 or Pep5 solution was weaker than that of the 1:2 mixture. In contrast, none of the polypeptides or the mixture exhibited a signal characteristic of an α-helical in the absence of Eu^{3+} ions.

The oligomerization states of the Pep4/Pep5/Eu^{3+} complex was determined by a sedimentation equilibrium centrifugation analysis (Fig. 5a). The analyzed data were fitted to a single species. The residuals were random and centered around zero, indicating that the complex is a single homogeneous species. The apparent molecular size of the complex was 11889 ± 361 daltons. This result shows that the Pep4/Pep5/Eu^{3+} complex forms a trimerized complex (the calculated molecular mass for Pep4/$(Pep5)_2$/Eu^{3+} is 12120 daltons). Furthermore, the Pep4/Pep5/Eu^{3+} complex was analyzed by using size exclusion chromatography with Sephadex G-50. The mixture

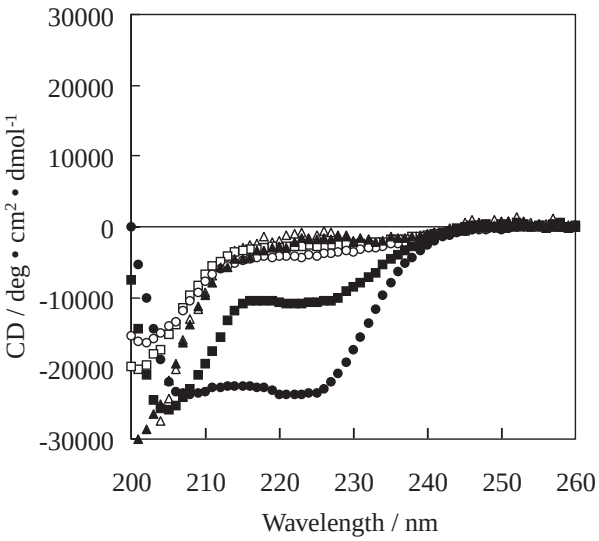

Fig. 4. Circular dichroism spectra of Pep4 (squares), Pep5 (triangles), and a 1:2 mixture of Pep4/Pep5 (circles) in the absence (open symbols) and presence (closed symbols) of 40 μM of $EuCl_3$. The measurements were performed in 10 mM Tris-HCl buffer containing 0.1 M NaCl (pH 7.0) at 20 °C. The polypeptide concentrations were 40 μM.

of Pep4/Pep5 showed a fraction corresponding to the trimer in the presence of Eu^{3+} ions (Fig. 5b). On the other hand, Pep 4 or Pep5 only showed a fraction corresponding to the monomer in spite of the existence of Eu^{3+} ions. These results indicate that the mixture of Pep4/Pep5 coordinate Eu^{3+} ions efficiently and form well-packed coiled-coil trimer complex. To determine the composition of the trimer from the Pep4/Pep5 mixture, HPLC analysis was performed. The eluted fraction of the trimer contained Pep4 and Pep5 in the ratio of 1.1:2.0 after normalization for the extinction coefficients (Fig. 5c). These results suggest that Pep4 and Pep5 formed the stable α-helical coiled-coil heterotrimer in the presence of Eu^{3+} ions.

The lanthanide ion-selective structural changes described above are applicable to new sensing systems. Therefore, for the purpose of designing direct rare metal ion detecting systems, the emission spectra of coordinated Eu^{3+} in the hydrophobic core of α-helical coiled-coil structure was measured. Emission spectrum of the Pep4/Pep5/Eu^{3+} complex is showed in Fig. 6a. Light excitation into the Trp $\pi\pi^*$ state at 280 nm was followed by strong red luminescence with a band maximum at around 619 nm. Furthermore, some emission bands were observed between 550 to 700 nm. These bands are characteristic of the f–f emission of Eu^{3+}. The excitation spectrum of the complex showed a peak at around 280 nm (Fig. 6b), which matches the corresponding absorption spectrum. These results confirm that the stable and effective coor-

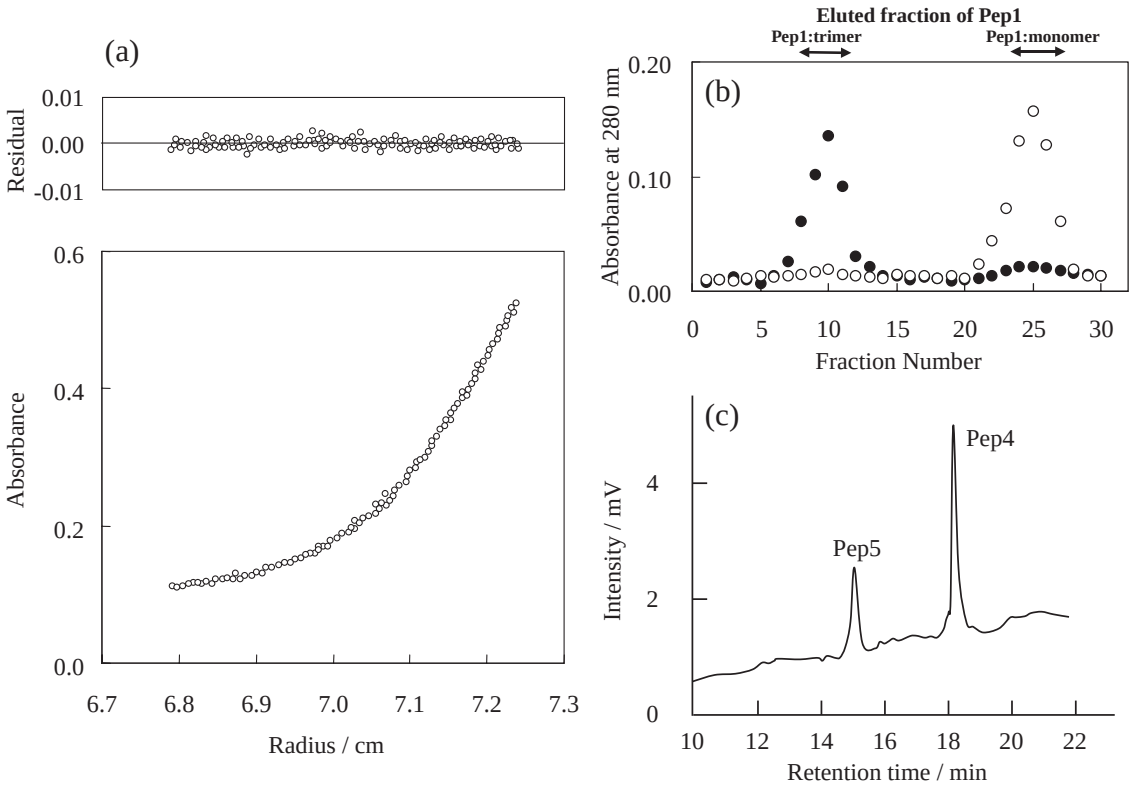

Fig. 5. (a) Sedimentation equilibrium analysis of the mixture of Pep4/Pep5/Eu^{3+} in 10 mM Tris-HCl buffer (pH 7.0) at 20 °C. (b) Analysis of the eluted fraction of the mixture of Pep4/Pep5 in the presence (●) or absence (○) of Eu^{3+} using Sephadex G-50 column. The elution was performed in 10 mM Tris-HCl buffer (pH 7.0) at 20 °C. The arrows indicate the eluted position of the standards, Pep1 in 10 mM Tris-HCl buffer (trimer) and Pep1 in 6 M guanidine hydrochloride solution (monomer). (c) HPLC profile of the major peak sample in elution of gel filtration chromatography with linear gradient of 45–55% CH_3CN containing 0.1% (v/v) TFA for 30 min.

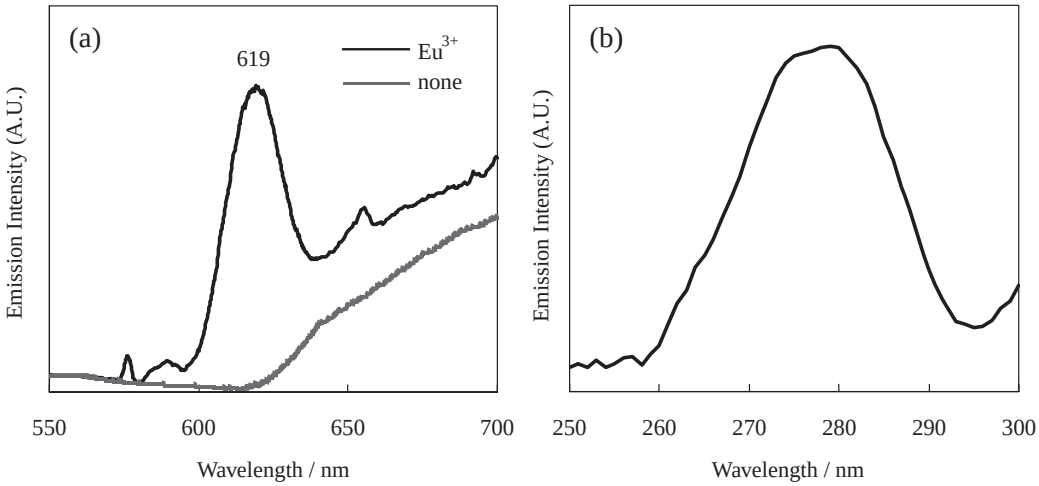

Fig. 6. (a) Emission spectra of the Pep4/Pep5 (1:2) mixture in the absence and presence of 40 μM of $EuCl_3$ with the excitation wavelength at 280 nm. (b) Excitation spectrum of the Pep4/Pep5 (1:2) mixture in the presence of 40 μM of $EuCl_3$ with the emission wavelength at 619 nm. The measurements were performed in 10 mM Tris-HCl buffer containing 0.1 M NaCl (pH 7.0) at −5 °C. The polypeptide concentrations were 40 μM.

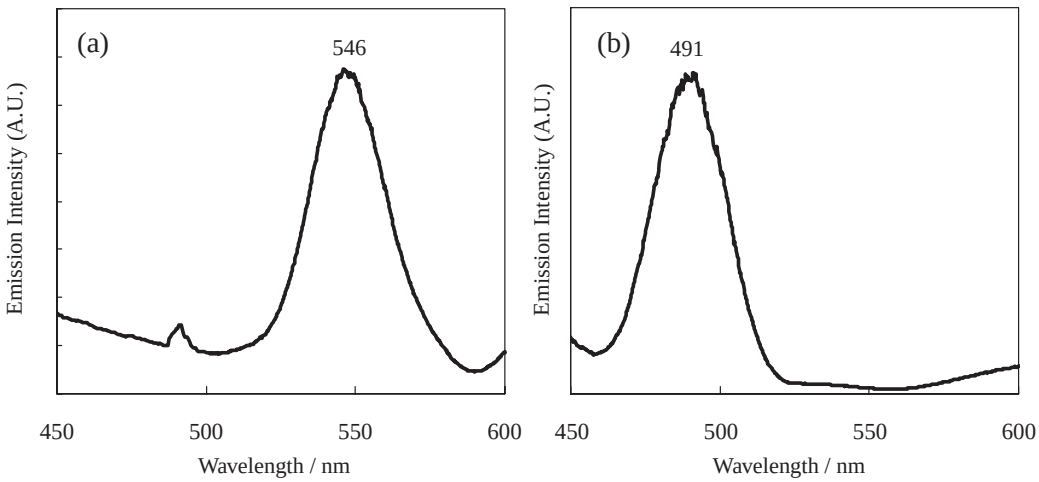

Fig. 7. Emission spectra of the Pep4/Pep5 (1:2) mixture in the presence of 40 μM of $TbCl_3$ (a) and $CeCl_3$ (b) with the excitation wavelength at 280 nm. The measurements were performed in 10 mM Tris-HCl buffer containing 0.1 M NaCl (pH 7.0) at −5 °C. The polypeptide concentrations were 40 μM.

dination from γ-Gla residue induced the energy transfer from the Trp layer to the Eu^{3+} ion in the complex. Emission spectra of Tb^{3+} and Ce^{3+} in the presence of the Pep4/Pep5 (1:2) mixture were also measured in a similar manner. As shown in Fig. 7, the polypeptide complex from Tb^{3+} and Ce^{3+} gave green (Em = 546 nm) and turquoise (Em = 491 nm) luminescences, respectively.

In this paper, we constructed a novel lanthanide ion-assembled system by using a γ-Gla including coiled-coil polypeptide, Pep3. Furthermore, two kinds of polypeptides, Pep4 and Pep5, underwent lanthanide ion-induced self-assembly into a heterotrimeric coiled-coil structure. Pep4 and Pep5 can mediate the associations of attached functional domains, in response to lanthanide ions. From the CD data, Eu^{3+} ions showed the highest affinity for complex formation. Based on the structural information, we constructed an efficient fluorescence sensing system that can be switched on by lanthanide ions-induced coiled-coil assembly. The detection of lanthanide characteristic f–f emission band by the steric conformational change of polypeptides is applicable for a highly sensitive lanthanide ion sensing systems.

Materials and Methods

Peptide Synthesis. All polypeptides used in this study were synthesized by the solid-phase synthesis method using Rink amide resin, N^{α}Fmoc-protected amino acids, HBTU, and HOBt. Deprotection and cleavage of polypeptides from the resin were performed by treatment with TFA/1,2-ethanedithiol/anisole/ethyl methyl sulfide (93/1/3/3, v/v) for 2 h. Following the cleavage reaction, polypeptides were purified by reverse phase HPLC on a YMC-Pack ODS-A column (10 mm i.d. × 250 mm, 5 μm, YMC Inc., Japan) eluted at 4 cm^3 min^{-1}. with linear acetonitrile/water gradients containing 0.1% (v/v) TFA over the course of 30 min. The final products were characterized by analytical HPLC and MALDI-TOF mass spectrometry, *m/z*: 3975 for Pep1 (calculated 3975); 4023 for Pep2 (calculated 4023); 3993 for Pep3 (calculated

3994); 4066 for Pep4 (calculated 4066); 3951 for Pep5 (calculated 3951).

Circular Dichroism (CD) Measurements. All CD measurements were performed on a Jasco J-820 spectropolarimeter using a 2 mm path length cuvette. Polypeptide concentration was determined from the tyrosine absorbance at 275 nm in 6 M (1 M = 1 mol dm^{-3}) guanidine hydrochloride solutions.[29] The mean residue ellipticity, $[\theta]$, is given in deg cm^2 $dmol^{-1}$.

CD spectra were measured in 10 mM Tris-HCl buffer containing 0.1 M sodium chloride (pH 7.0). The polypeptide concentrations were 40 μM.

Sedimentation Equilibrium. Sedimentation equilibrium analysis was performed with a Beckman XL-I Optima Analytical Ultracentrifuge equipped with absorbance optics. The polypeptide samples were dissolved in Tris-HCl buffer (10 mM, pH 7.0, containing 0.1 M sodium chloride). The polypeptide concentration was 60 μM. The samples were centrifuged at 30000 r.p.m. at 20 °C, and the absorbance was monitored at 230 nm. A partial specific volume of 0.760 was used, which was obtained from the mass average of the partial specific volumes of the individual amino acids. The oligomerization state was determined by fitting the data to a single species, using Origin Sedimentation Equilibrium Single Data Set Analysis (Beckman).

Size Exclusion Gel Filtration Chromatography. The polypeptide samples were dissolved in 0.15 cm^3 of Tris-HCl buffer (10 mM, pH 7.0, containing 0.1 M sodium chloride). The sample solutions were added to a Sephadex G-50 column (6.0 i.d. × 110 mm) and were eluted with the same buffer. The eluted solution was collected in 0.1 cm^3 samples and monitored at a wavelength of 280 nm. The total polypeptide concentration was 80 μM. The main peak in elution of gel filtration chromatography was analyzed by reverse phase HPLC on a YMC-Pack ODS-A column (4.6 mm i.d. × 250 mm, 5 μm, YMC Inc.) eluted at 1 cm^3 min^{-1} with linear acetonitrile/water gradients containing 0.1% (v/v) TFA over the course of 30 min. Concentrations of polypeptides were measured using ε_{275} value.

Luminescence Measurement. Excitation and emission spectra were measured in 10 mM Tris-HCl buffer of pH 7.0 containing 100 mM NaCl on a Hitachi F4500 fluorescence spectrophotometer with. All fluorescence measurements were carried out with 3 cm^3 of sample solution in a 1 cm pathlength quartz cuvette.

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