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Monodispersed Dimerization of Isoleucine Zipper–Coiled Coil Trimer

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We describe the synthesis and characterization of novel isoleucine zipper polypeptide dimers, connected with a maleimide-containing linker molecule, that can assemble into structurally defined heterotrimeric α-helical coiled coil dimers on the basis of architectural features of the polypeptide sequences. Linear- and crosslinked-isoleucine zipper polypeptide dimers were designed in this study. Circular dichroism spectroscopy, gel filtration, and HPLC analyses indicate that each polypeptide dimer can noncovalently assemble with four isoleucine zipper polypeptides and lead to heterotrimeric α-helical coiled coil dimer formation. Thus, we concluded that monodispersed dimerization of triple-stranded coiled coil was achieved for the first time. Moreover, the noncovalently assembled supramolecule may be useful as a the building-block for constructing artificial polypeptide fibrillogenesis systems.

Noncovalent interactions of native proteins play an important role in supramolecular assembly and biological activity. Therefore, there is great interest in the rational design of self-assembled polypeptides. Among these designed polypeptides, fibrous polypeptides are important motifs for biological and nanotechnological applications. Up to now, several polypeptides including α-helix and β-sheet structure units have been designed for constructing well-defined fibrous supramolecular assemblies. Although self-assembling fibrils derived from β-sheet motifs have been widely investigated as nanoarchitecture,[1–4] the design of α-helical-assembled fibrils is not as advanced.[5–7]

α-Helical scaffolds differ in critical structural features such as the periodic long axis of the fibril, from β-sheet assemblies. This suggests that helical protomers are considered as complementary modules to β-strands for the development of functional nanoscale materials. Moreover, in contrast to amyloidogenic polypeptides, the principles that govern the association of α-helical protomers into discrete coiled coil assemblies[8] have been elucidated in detail from structural studies on model polypeptides, such as leucine zippers[9–12] and isoleucine zippers.[10,11,13] However, the potential of these α-helical coiled coil polypeptides for the construction of well-defined fibril structures remains largely untapped in spite of the fact that fibrious structures derived from α-helical coiled coil motifs are formed widely in native biological systems, such as cytoskeleton and extracellular matrix.[14] Thus, α-helical motifs are an attractive target for de novo design of well-defined fibrils from self-assembly of synthetic helical protomers.

We have reported an AAB-type-heterotrimeric α-helical coiled coil by mutation of one amino acid residue at the hydrophobic *a* position of an isoleucine zipper polypeptide.[15–17] In our design, two kinds of amino acids with different side chain sizes have been used. One Ala residue at the hydrophobic *a* position destabilizes the formation of the coiled coil trimer, because of the small size of its side chain. On the other hand, Trp residue is thought to cause steric hindrance, in the case where three Trp residues are oriented toward the same hydrophobic core. However, if the complexes can form the formation of 2:1 Ala–Trp layers at the three consecutive *a* position can also form. In this study, we will report the synthesis and characterization of linear- and crosslinked-isoleucine zipper polypeptide dimers (LD-IZW and CLD-IZW, respectively) (Fig. 1), to expand the utility of our approach to the facile assembly of discrete multivalent supramolecules in aqueous solutions. These polypeptide dimers were prepared in a similar manner to IZW polypeptide, which has Trp_{17} at the central core *a* position of isoleucine zipper polypeptide (Pep1). Due to the steric hindrance of a bulky Trp side chain and poor flexibility of polypeptides by linker-connection, LD-IZW or CLD-IZW should not self-assemble. However, these dimers are expected to assemble with four IZA polypeptides, which have Ala_{17} at the central core *a* position of Pep1. As a result, heterotrimeric α-helical coiled coil dimer structures were constructed. Such a system should have great utility in the monodisperse and fibrillar noncovalent supramolecular assemblies that span the nanometer to micrometer regime.

Results and Discussion

We designed LD-IZW and CLD-IZW to assemble with and form heterotrimeric α-helical coiled coil dimer structures when mixed with 4 equivalents of an IZA polypeptide. The substructure of this design had AAB-type-heterotrimeric α-helical coiled structures. We utilized a linker molecule **2** (Fig. 1) with maleimide groups on both ends to design and synthesize the polypeptide dimers. Maleimide is known for

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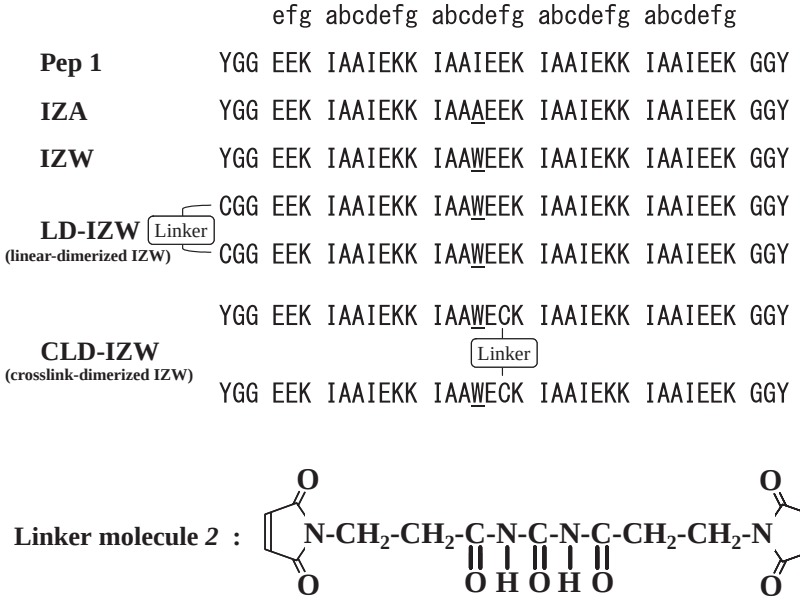

Fig. 1. Amino acid sequences of isoleucine zipper coiled coil polypeptides used in this study.

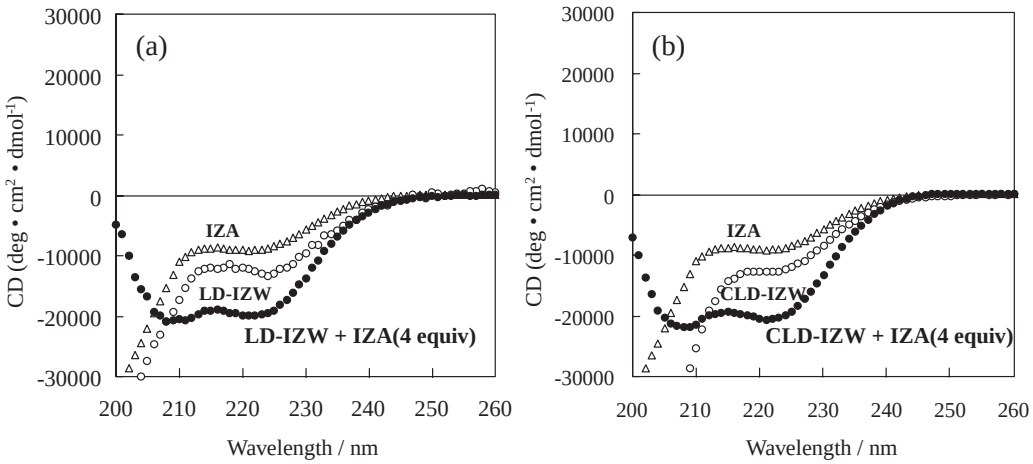

Fig. 2. CD spectra of LD-IZW/4IZA and CLD-IZW/4IZA assembled at pH 7.0 and 20 °C. (a) CD spectra of IZA (△), LD-IZW (○), and LD-IZW/IZA (1:4) mixture (●). (b) CD spectra of IZA (△), CLD-IZW (○), and CLD-IZW/IZA (1:4) mixture (●).

chemoselective covalent tethering to thiol-containing molecules. Therefore, we tried to couple the IZWs containing Cys residues using linker molecule **2**. Since LD-IZW had a Cys_1 residues at the N-terminal and CLD-IZW polypeptide has Cys_{19} at the central *f* position as hydrophilic exterior wall, respectively, these Cys-substitutions should have no influence on the formation of the α-helical coiled coil forming.

Circular dichroism (CD) spectroscopy was utilized for characterizing the secondary structure of the polypeptides designed here. The CD spectra showed that LD-IZW and IZA had a random structure. On the other hand, LD-IZW with 4 equivalents of IZA showed minimum molar elipticities at 208 and 222 nm, which is characteristic of an α-helical structure (Fig. 2a). If there is no interaction between LD-IZW and IZA, then the spectrum of LD-IZW with IZA would be equivalent to the sum of the spectra for 6.7 μM of LD-IZW and 27.7 μM of IZA. However, the CD spectrum of LD-IZW/IZA (6.7 μM/27.7 μM) mixture was more characteristic of an α-helical structure than the sum of the spectra. This spectral change indicates that LD-IZW interacts with IZA and forms a linear-heterotrimeric α-helical coiled coil dimer structure. Similarly, CLD-IZW also formed an α-helical structure in the presence of IZA (Fig. 2b), indicating the formation of a crosslinked-heterotrimeric α-helical coiled coil dimer structure. Figure 3 shows the CD titration profiles of 6.7 μM of LD-IZW (Fig. 3a) and CLD-IZW (Fig. 3b) with the concentration of IZA. In the titration curves of both LD-IZW and CLD-IZW, the θ_{222} value increased with the IZA concentration and then stayed almost constant in the presence of an excess amount of IZA. Therefore, the bending point at an IZA concentration of 27.7 μM indicates that LD-IZW or CLD-IZW retained 4 equivalents (27.7 μM) of IZA to form a stable complex. These titration experiments confirmed that design of self-organized heterotrimeric α-helical coiled coil dimer was achieved.

We also measured the fluorescence spectra of Trp residues in LD-IZW or CLD-IZW to clarify the environment of the in-

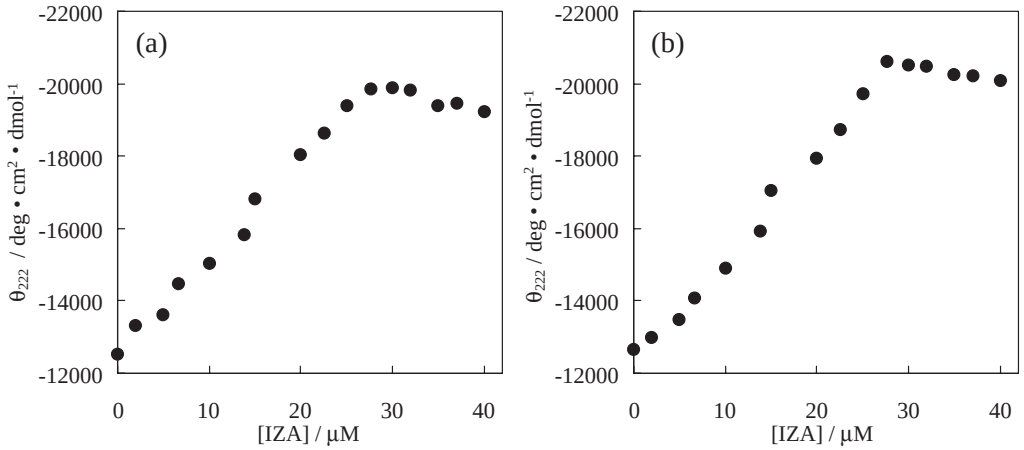

Fig. 3. IZA titration profile of LD-IZW (a) and CLD-IZW (b) monitored by CD spectroscopy at pH 7.0 and 20 °C. $[\theta]_{222}$ was monitored and plotted as a function of IZA. The concentration of LD-IZW or CLD-IZW was 6.7 μM.

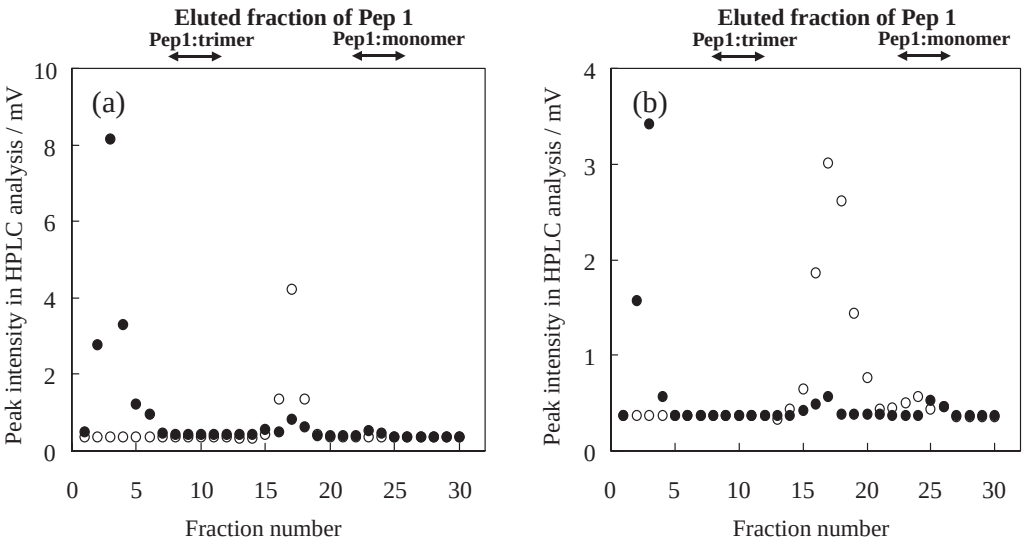

Fig. 4. Analysis of the eluted fraction of LD-IZW (a) and CLD-IZW (b) in the presence (●) or absence (○) of IZA using Sephadex G-50 column. The elution was performed in 10 mM Tris-HCl buffer (pH 7.0) at 20 °C. The arrows indicate the eluted position of the standards, Pep1 in 10 mM Tris-HCl buffer (trimer) and Pep1 in 6 M guanidine hydrochloride solution (monomer).

dole side chain. LD-IZW/$(IZA)_4$ and CLD-IZW/$(IZA)_4$ mixture exhibited fluorescence maxima of the Trp residues at 330 and 333 nm, respectively. On the other hand, both LD-IZW and CLD-IZW gave fluorescence maxima at 351 nm in the absence of IZA. These results support the formation of AAB-type-heterotrimeric α-helical coiled coil.[15]

The oligomerization of polypeptide was determined by using Sephadex G-50 gel filtration chromatography (Fig. 4). In the absence of IZA, both LD-IZW and CLD-IZW eluted as a fraction corresponding to a dimer, judging from the elution of the standard, Pep1, which has a similar amino acid length. Furthermore, we used sedimentation equilibrium centrifugation analysis to determine the oligomerization state of the LD-IZW/IZA and CLD-IZW/IZA complexes. The apparent molecular sizes of these polypeptide complexes were 24419 ± 479 and 23899 ± 602 daltons, respectively (Fig. 5), indicating that LD-IZW and CLD-IZW formed a monodispersed complex with 4 equivalents of IZA: the calculated molecular masses for the LD-IZW/$(IZA)_4$ and CLD-IZW/$(IZA)_4$ are 24074 and 24142 daltons respectively.

The compositions of the resultant complexes of the LD-IZW or CLD-IZW with 4 equivalents of IZA mixture were analyzed. Both polypeptide mixtures eluted quicker than the Pep1. To analyze the stoichiometry of the resultant polypeptide complex from the LD-IZW and 4 equivalents of IZA, HPLC analysis was used, and the chromatogram is shown in Fig. 6a. The eluted fraction of the polypeptide complex contained LD-IZW and IZA in a ratio of 1.0:3.9 after normalization of the extinction coefficients. In the same way, the composition of the polypeptide complex involving CLD-IZW and the IZA was estimated to be 1.0:4.0 (Fig. 6b). These results also suggest that LD-IZW or CLD-IZW can noncovalently assemble four IZA molecules.

Relative stabilities of heterotrimeric α-helical coiled coil dimers were established by using chemical denaturation with urea. Unfolding free energies (ΔG°) at 20 °C were calculated by least-squares fitting to a two state equilibrium between monomer and trimer (See the experimental section). The con-

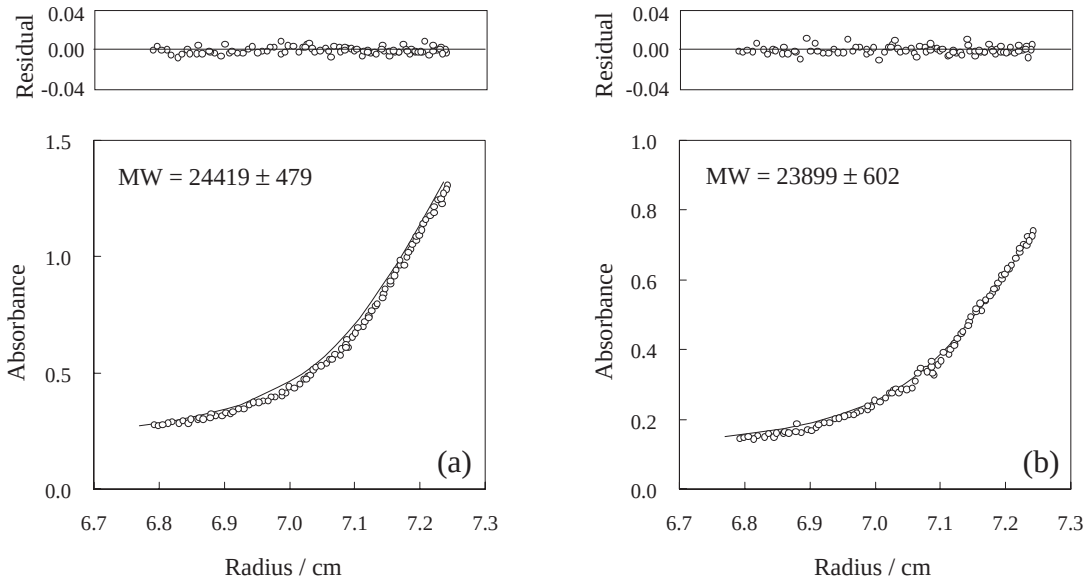

Fig. 5. Sedimentation equilibrium analysis of the mixture of LD-IZW/4IZA (a) and CLD-IZW/4IZA (b) in 10 mM Tris-HCl buffer (pH 7.0) at 25 °C.

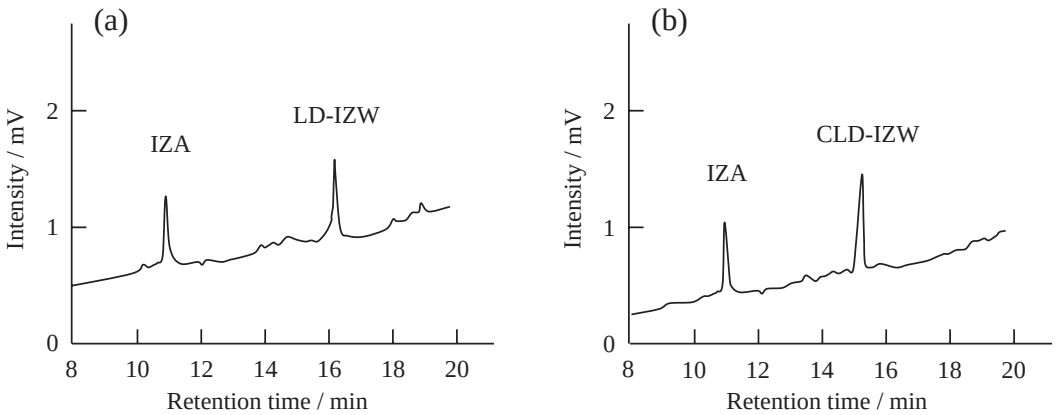

Fig. 6. HPLC profile of the major peak sample in elution of gel filtration chromatography with linear gradient of 50–65% CH_3CN containing 0.1% (v/v) TFA for 30 min. (a) LD-IZW/IZA mixture and (b) CLD-IZW/IZA mixture.

trol Pep1, which forms a stable α-helical coiled coil trimer structure,[13] has an urea denaturation midpoint of 2.1 M, and ΔG° was calculated to be 110 kJ mol^{-1}. LD-IZW and CLD-IZW were only about 21.1 and 17.4% folded at pH 7.0 and 20 °C, respectively. These linear- and crosslinked-dimers had ΔG° values of 55.6 and 52.7 kJ mol^{-1}, respectively. On the other hand, they were fully folded with 4 equivalents of IZA, and urea denaturation yielded ΔG° values of 100 and 102 kJ mol^{-1}, respectively (Fig. 7). Thus, each polypeptide dimer are stabilized by about 42 kJ mol^{-1} upon coiled coil formation with IZA. Furthermore, the resultant heterotrimeric α-helical coiled coil dimers show similar stability as that of the control Pep1. Moreover, the thermodynamic stabilities of LD-IZW and CLD-IZW with 4 equivalents of IZA were obtained by measuring the variation of $[\theta]_{222}$ at different temperatures. In LD-IZW and CLD-IZW, reasonable values were obtained for the melting temperature ($T_m = 52.7$ and 50.1 °C, respectively) in comparison to control Pep1 ($T_m = 57.7$ °C) and similar coiled coil model shown in previous papers.[6,15,16] The results of thermodynamical characterizations mentioned above show that structurally defined and monodispersed building blocks can be used to construct supramolecular fibrious structures.

In this work, a noncovalent dimeric assemblies of heterotrimeric α-helical coiled coil were prepared. Furthermore, we also showed that linker molecule **2** is effective for stabilizing the formation of noncovalent assembly of cognate polypeptides. This approach for the assembly of α-helical structure makes the preparation of novel self-organizing bio- and nano-materials via different polypeptide sequences and linker structures.

Experimental

Peptide Synthesis. All polypeptides used in this study were synthesized by using a solid-phase synthesis method according to standard fluorenylmethoxycarbonyl (Fmoc) strategy starting with Rink amide resin. Fmoc amino acids were chemically activated with HBTU (*N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methylmethanaminiumhexafluorophosphate *N*-oxide) in the presence of 1-hydroxybenzotriazole (HOBt) and *N*,*N*′-diisopropylethylamine (DIEA).[18] Deprotection and cleavage

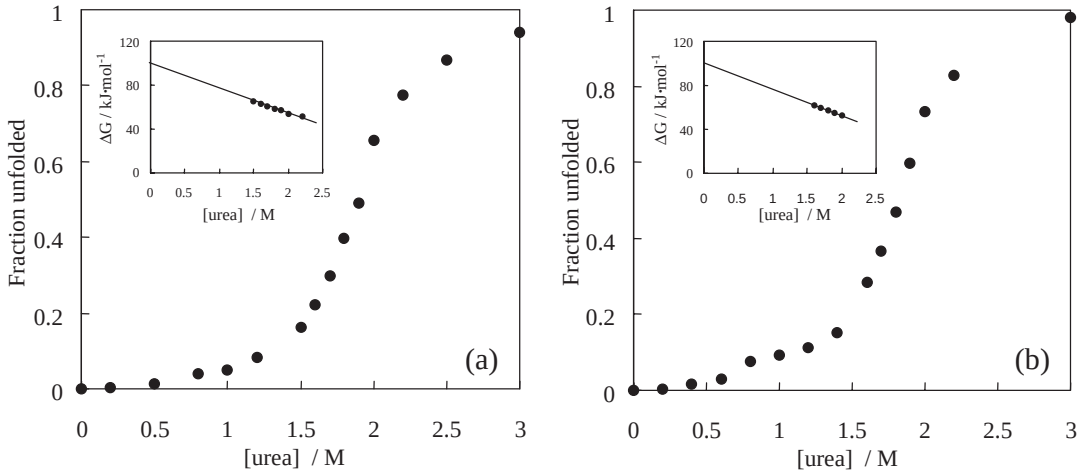

Fig. 7. Urea denaturation profiles of LD-IZW/IZA (a) and CLD-IZW/IZA (b) in 10 mM Tris-HCl buffer (pH 7.0) at 20 °C. The insets show the linear dependence of ΔG on the urea concentration. ΔG is the free energy of unfolding at a given concentration of urea.

of polypeptides from the resin were performed by treating with TFA (trifluoroacetic acid)/1,2-ethanedithiol/anisole/ethyl methyl sulfide (volumetric ratio: 93/1/3/3) for 2 h. After the cleavage reaction, the polypeptides were purified by a 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 vol % TFA over the course of 30 min. The final products were characterized by using analytical HPLC and MALDI-TOF mass spectrometry, m/z: 3971 for Pep1 (calculated 3971); 3935 for IZA (calculated 3935); 3992 for IZW1 (calculated 3990); 4024 for IZW2 (calculated 4024).

Synthesis of *N*-(3-Maleimidopropanoyl)succinimide (1). *N*-(3-Maleimidopropanoyl)succinimide (**1**), the starting material for linker molecule **2**, was synthesized according to previous paper.[19]

Synthesis of Linker Molecule 2. First, **1** (2.0 mg, 0.75 mmol) was dissolved in DMSO (1.0 cm^3). Then, to the solution DIEA (1.0 mg, 0.78 mmol) was added. After 2 min, the solution of **1** was added into urea solution (0.12 mg (2.0 mmol) urea/1.0 cm^3 DMSO). The reaction mixture was shaken at room temperature for 4 h. The resultant solution was acidified with acetic acid and purified by using a reverse-phase HPLC on a YMC-Pack ODS-A column (4.6 mm i.d. × 250 mm, 5 μm, YMC Inc., Japan) eluted at 1 cm^3 min^{-1} with 60% acetonitrile solution over the course of 30 min. The final products were characterized by using ^{1}H NMR spectrometry and organic elements analysis. ^{1}H NMR (270 MHz, DMSO-d_6) δ 8.10 (s, 2H, NH), 7.04 (s, 4H, maleimido CH), 3.71 (t, 4H, CH_2), 2.33 (t, 4H, CH_2). Anal. Calcd for $C_{15}H_{14}N_4O_7$: C, 49.73; H, 3.89; N, 15.47%. Found: C, 50.04; H, 4.01; N, 15.52%.

Synthesis of Peptide Dimer. LD-IZW was synthesized from 10 mg of purified IZW1, which had Cys residue at the N-terminal, in 3.0 cm^3 of triethylamine/acetic acid buffer (pH 7.0). Then, **2** (2.0 mg, 0.55 mmol) in DMSO (0.3 cm^3) was added to the solution. The reaction mixture was gently stirred at 35 °C for 10 h. The product was purified by using dialysis and gel filtration chromatography. Further purification was performed by using 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. The final products were characterized by MALDI-TOF mass spectrometry, m/z: 8336 (calculated 8334 for LD-IZW).

Another polypeptide dimer, CLD-IZW was synthesized from 10 mg of purified IZW2, which had a Cys residue in the middle. The synthetic procedure was the same as above. The final products were characterized by using MALDI-TOF mass spectrometry, m/z: 8401 (calculated 8402 for CLD-IZW).

CD Measurements. All CD measurements were performed on a Jasco J-820 spectropolarimeter equipped with a 2 mm pathlength cuvette. The polypeptide concentration was determined from the tyrosine absorbance at 275 nm in a 6 M guanidine hydrochloride solution.[20] θ is given in deg cm^2 $dmol^{-1}$. CD spectra were measured in a 10 mM Tris-HCl buffer (pH 7.0) containing 0.1 M sodium chloride.

Titration of LD-IZW or CLD-IZW with IZA was carried out in the same buffer by monitoring $[\theta]_{222}$ as a function of the IZA concentration. The concentration of LD-IZW or CLD-IZW was 6.7 μM.

The urea denaturation curve, which was developed from $[\theta]_{222}$, was obtained for the polypeptide mixtures (6.7 μM of LD-IZW or CLD-IZW and 26.7 μM of IZA). The results of denaturation experiments were analyzed according to a nonlinear least-squares fitting procedure. The two-state model expressed in Eq. 1 was used.

$$\begin{aligned}&[\text{LD-IZW}] + 4[\text{IZA}] \rightleftharpoons [\text{LD-IZW}\cdot(\text{IZA})_4] \text{ or}\\ &[\text{CLD-IZW}] + 4[\text{IZA}] \rightleftharpoons [\text{CLD-IZW}\cdot(\text{IZA})_4].\end{aligned} \quad (1)$$

ΔG° was estimated from the linear extrapolation, according to $\Delta G = \Delta G^\circ - m[\text{urea}]$.[21]

The thermal transition was monitored $[\theta]_{222}$ against the temperature with a 10 mm pathlength cuvette. The temperature was increased at a rate of 0.5 °C min^{-1}.

Tryptophan Fluorescence Spectroscopy. Tryptophan fluorescence measurements were carried out at 25 °C on a HITACHI F-4500 spectrofluorometer with a 1 cm pathlength cuvette. The emission spectra of tryptophan were measured with excitation at 278 nm. The polypeptide samples were dissolved in Tris-HCl buffer (10 mM, pH 7.0, containing 0.1 M sodium chloride). The polypeptide concentration was 40 μM.

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 applied to a Sephadex G-50 column (6.0 (i.d.) ×

110 mm) and were eluted with the same buffer. An eluent of 0.1 cm^3 was collected 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_{280} value (5700 for Trp and 1300 for Tyr).

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 rpm at 25 °C, and the absorbance was monitored at 230 nm. The oligomerization state was determined by fitting the data to a single species, using Origin Sedimentation Equilibrium Single Data Set Analysis (Beckman).

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