Design of a Heterotrimeric α-Helical Bundle by Hydrophobic Core Engineering

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Abstract: We previously prepared the de novo designed peptide, [YGG(IEKKIEA)$_4$], which forms a parallel triple-stranded coiled coil. To prepare an AAB-type heterotrimeric α-helical bundle, two variants, where the Ile[15] residue in the hydrophobic position was replaced with either an Ala or Trp residue, were designed and named IZ-2A and IZ-2W, respectively. Circular dichroism spectroscopy, peptide titration, sedimentation equilibrium, and gel filtration analyses revealed the formation of an (IZ-2A)$_2$/IZ-2W complex. The NOESY spectra analyses indicated the presence of interstrand interactions between the two Ala residues and the Trp residue in the hydrophobic core. The (IZ-2A)$_2$/IZ-2W complex has the structural uniqueness of native proteins.

Introduction

The coiled coil is often observed in natural proteins for intermolecular assemblies of the functional domains. This motif has been the subject of extensive analyses to understand the principles of de novo design, as well as of protein folding and stability.[1] It has the representative amino acid sequence of (defgabc)$_n$ heptad repeats. The a and d positions are usually occupied by hydrophobic residues and form the hydrophobic core. One or two amino acid substitutions in the hydrophobic core can function to determine the structural specificity,[2] to control the oligomerization,[3] and to induce the pH- or metal ion-dependent folding.[4] The amino acids in the hydrophobic core should have more influence on the structure than those at the other positions.

ABC type heterotrimers have been constructed, where the ionic interactions at the e and g positions were employed to mediate heterospecificity.[5] Here we report a new design for an AAB type heterotrimeric α-helical bundle, by the substitution of one amino acid at a hydrophobic position. We used a side-chain with a complementary size to engineer the specificity in the packing of the hydrophobic core of the coiled coil.

We have designed a parallel triple-stranded coiled coil with the amino acid sequence of YGG(IEKKIEA)$_4$ (defgabc), IZ.[6] We replaced the Ile residue at the a position in the second heptad repeat with either an Ala or a Trp residue. These peptides are designated as IZ-2A and IZ-2W, respectively. Due to the small side chain of the Ala residue, the homotrimeric structure of (IZ-2A)$_3$ should be destabilized. On the other hand, if the three Trp side chains are oriented toward the inside of the coiled coil, then they should produce steric hindrance in (IZ-2W)$_3$. The bulky Trp side chain is expected to compensate for the hole in the hydrophobic core created by the two Ala residues from the two other peptide strands.

Results and Discussion

The circular dichroism (CD)[7] spectrum of IZ-2A exhibited an almost random structure with a minimum at 200 nm (Figure 1). On the other hand, that of IZ-2W revealed an α-helical structure with minima at 208 and 222 nm. A mixture of IZ-2A/IZ-2W (2:1) gave a CD spectrum similar to that of IZ-2W. However, the [θ] value of the IZ-2A/IZ-2W (2:1) mixture was higher than that of IZ-2W, despite the presence of IZ-2A. It suggests that IZ-2A formed the α-helical structure and interacted with IZ-2W. Figure 2 shows the IZ-2W titration profile of IZ-2A monitored by CD spectroscopy. The $[\theta]_{222}$ value increased until an IZ-2A/IZ-2W ratio of 2, and then was almost a plateau, suggesting that IZ-2A and IZ-2W interacted in a 2:1 ratio.

The peptide oligomerization was determined by a sedimentation equilibrium centrifugation analysis. IZ-2W and a mixture of IZ-2A/IZ-2W (2:1) at a 20 μM concentration gave apparent molecular sizes of 10 221 and 10 089 Da, respectively, indicat-

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(7) Abbreviations: RP-HPLC, reversed phase high performance liquid chromatography; CD, circular dichroism; NOE, nuclear Overhauser effect; DQF-COSY, double quantum filtered correlation spectroscopy.

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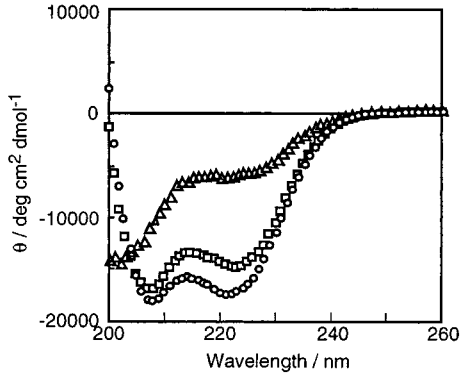

Figure 1. CD spectra of IZ-2A, IZ-2W, and the IZ-2A/IZ-2W (2:1) mixture at 20 °C and pH 7.0. The peptide concentrations were 20 μM. (△) IZ-2A, (□) IZ-2W, (○) IZ-2A/IZ-2W (2:1) mixture.

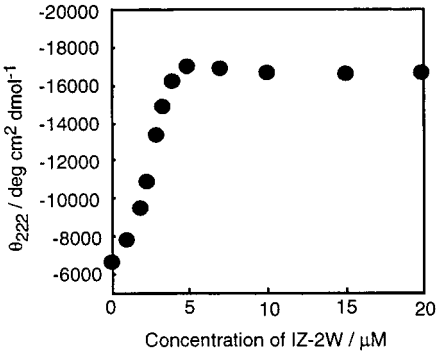

Figure 2. IZ-2W titration profile of IZ-2A monitored by CD spectroscopy at 20 °C and pH 7.0. The $[\theta]_{222}$ was monitored and plotted as a function of IZ-2W. The IZ-2A concentration was 10 μM.

ing that both peptides were trimerized (the calculated molecular masses for $(IZ\text{-}2W)_3$ and $(IZ\text{-}2A)_2/IZ\text{-}2W$ are 10 899 and 10 669 Da, respectively).[8] Moreover, we analyzed their complexes by Sephadex G-50 gel filtration chromatography. The peptides were eluted at the fraction corresponding to a trimerized peptide. In the case of a mixture of IZ-2A/IZ-2W, the eluted fraction, when analyzed by RP-HPLC, contained IZ-2A and IZ-2W in a ratio of 1.9:1 after normalization for the extinction coefficients.[9] These results also show that the $(IZ\text{-}2A)_2/IZ\text{-}2W$ complex was formed in the solution. Thermal denaturation was carried out using $(IZ\text{-}2A)_2/IZ\text{-}2W$ and $(IZ\text{-}2W)_3$ and revealed transitions with melting temperatures of 60 and 51 °C, respectively (Figure 3). The transition curve of $(IZ\text{-}2A)_2/IZ\text{-}2W$ was more steep than that of $(IZ\text{-}2W)_3$. Thus, $(IZ\text{-}2A)_2/IZ\text{-}2W$ has higher thermal stability and a higher cooperative transition.

We designed a parallel orientation of the heterotrimer by using charge−charge interactions between the e and g positions. To analyze the helical orientation of the $(IZ\text{-}2A)_2/IZ\text{-}2W$ complex, IZ-3A was prepared. IZ-3A has Ala at the a position in the third heptad repeat of IZ. If the $(IZ\text{-}2A)_2/IZ\text{-}2W$ complex has an antiparallel orientation, then the $(IZ\text{-}3A)_2/IZ\text{-}2W$ complex should have higher stability. However, the $(IZ\text{-}3A)_2/IZ\text{-}2W$ complex was not observed in the mixture of IZ-3A and IZ-2W after the gel filtration followed by the HPLC analysis. This result suggests that the orientation of the three α-helices of the $(IZ\text{-}2A)_2/IZ\text{-}2W$ complex is parallel.

Trp fluorescence is sensitive to the environment of the indole side chain. The Trp residue shows fluorescence emission maxima at 327−332 nm in a hydrophobic environment and 354 nm in water.[10] The fluorescence maximum of the Trp residue in the $(IZ\text{-}2A)_2/IZ\text{-}2W$ complex was at 328 nm after excitation at 278 nm, indicating that the Trp residue in $(IZ\text{-}2A)_2/IZ\text{-}2W$ was completely buried in the complex. On the other hand, the $(IZ\text{-}2W)_3$ complex gave a fluorescence maximum at 340 nm, indicating that the Trp residues were either partially buried or in equilibrium between the buried and solvent exposed positions.

(8) See Supporting Information.
(9) See Supporting Information.

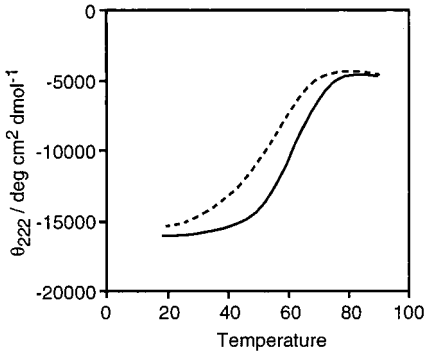

Figure 3. Thermal melting curves of $(IZ\text{-}2A)_2/IZ\text{-}2W$ and $(IZ\text{-}2W)_3$. Mean residue ellipticities at 222 nm of $(IZ\text{-}2A)_2/IZ\text{-}2W$ (solid line) and $(IZ\text{-}2W)_3$ (dashed line) are plotted as a function of temperature. The CD spectra were recorded in 10 mM sodium phosphate, pH 7.0. The total peptide concentrations were 20 μM for both peptides.

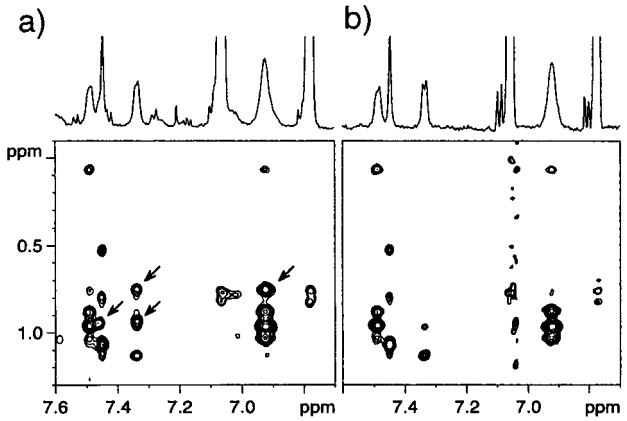

Figure 4. ^{1}H-NOESY spectrum of the $(IZ\text{-}2A)_2/IZ\text{-}2W$ complex in ^2H_2O at pH 7.0 and 25 °C. (a) The methyl-aromatic region of the NOESY spectrum of the $(IZ\text{-}2A)_2/IZ\text{-}2W$ complex. (b) The same region of the NOESY spectrum of the (IZ-2A incorporated with 3,3,3-$^2H_3$-Ala)$_2$/IZ-2W complex. The mixing time was 100 ms. The cross-peaks that disappeared are indicated by arrows in Figure 4a.

Figure 4 illustrates the aromatic region of the ^{1}H NMR and selected regions of the NOESY spectra of the $(IZ\text{-}2A)_2/IZ\text{-}2W$ complex at pH 7.0 and 25 °C. The aromatic protons were well dispersed and showed a single set of resonances. We first assigned the aromatic protons of the Trp residue, using a combination of DQF-COSY and NOESY spectra [11] as follows: 7.48 ppm, ϵ3-CH; 7.45 ppm, δ1-CH; 7.34 ppm, η2−CH; 6.94 ppm, ζ3-CH; 6.92 ppm, ζ2-CH. The two peaks at 7.18 and 6.78 ppm were ϵ-CH and δ-CH from a Tyr residue, respectively.

In the NOESY spectrum, many cross-peaks between aromatic protons and methyl protons were observed (Figure 4a). To clarify the interstrand contact between the Trp and Ala residues, we prepared IZ-2A, in which a 3,3,3-$^2H_3$-Ala residue was incorporated at the a position, and compared the NOESY

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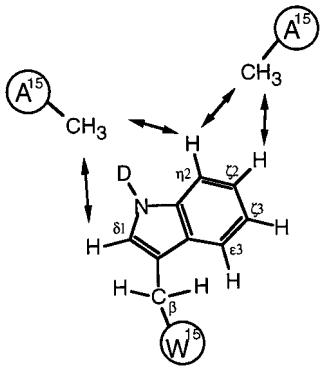

Figure 5. Observed interstrand interactions between the two Ala residues and the Trp residue in the $(IZ\text{-}2A)_2$/IZ-2W complex.

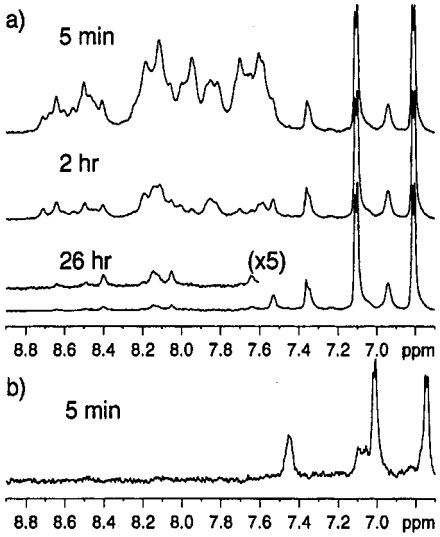

Figure 6. ^1H NMR amide hydrogen-exchange studies of (a) $(IZ\text{-}2A)_2$/IZ-2W and (b) $(IZ\text{-}2W)_3$. The time after addition of ^2H_2O is listed next to each spectrum. All spectra were recorded in ^2H_2O at pH 7.0. The total peptide concentration was 1 mM.

spectra. The two sets of cross-peaks from 0.75 and 0.93 ppm were absent, as shown in Figure 4b. These signals were, therefore, assigned as the Ala residues interacting with the Trp residue.[12] Judging from the cross-peaks, the methyl protons at 0.75 ppm were spatially close to η2−CH and ζ2-CH, and the methyl protons at 0.93 ppm were close to ζ2-CH and δ1-CH (Figure 5). These results clearly indicate that the Trp residue was placed inside the hydrophobic core and interacted with the two Ala residues, as designed.

Native proteins should have interior side chains with a unique conformation, which results in the presence of amide protons that are protected from hydrogen exchange in their NMR spectra.[13] We performed H/D exchange experiments at pH 7 (direct reading) and 25 °C. For the $(IZ\text{-}2A)_2$/IZ-2W complex, several amides remained visible even after 26 h (Figure 6). In contrast, the H/D exchange reaction was completed within 5 min in the $(IZ\text{-}2W)_3$ complex. This result indicates that $(IZ\text{-}2A)_2$/IZ-2W has a nativelike structure, while $(IZ\text{-}2W)_3$ is in a molten globule state.

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Conclusions

The AAB type heterotrimer was constructed by the manipulation of single Ala and Trp residues at the hydrophobic a position. In the $(IZ\text{-}2A)_2$/IZ-2W complex, the bulky indole side chain of the Trp residue fits snugly within the cavity created from the two Ala residues in the triple-stranded coiled coil. If another set of Ala and Trp residues is placed between the different strands, then an ABC type heterotrimer should be constructed. Besides the set of Ala and Trp residues, the selection of amino acids at the e and g positions should increase the selectivity of the AAB or ABC type of heterotrimer formation. This construct is useful not only to create de novo designed functional proteins but also to study protein−protein interactions.

Experimental Section

Peptide Synthesis and Purification. IZ-2A and IZ-2W were 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. 3,3,3-$^2H_3$-Ala was purchased from Cambridge Isotope Laboratories and was derivatized to fluorenylmethoxycarbonyl (Fmoc)-$^2H_3$-Ala. It was used for automatic peptide synthesis. The peptide was simultaneously cleaved/deprotected with trifluroacetic acid (TFA)/water (95:5 v/v) for IZ-2A and TFA/1,2-ethandithiol/water (95:2.5:2.5 v/v) for IZ-2W for 1.5 h, and was purified by RP-HPLC. Purification was carried out by reverse phase HPLC on a YMC-Pack ODS-A column (10 mm i.d. × 250 mm, 5 μm, YMC Inc., Japan) with a linear gradient of 35 to 45% 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*: 3518 for IZ-2A (calcd 3518); 3634 for IZ-2W (calcd 3633); 3521 for IZ-2A incorporated with 3,3,3-$^2H_3$-Ala (calcd 3521).

Circular Dichroism (CD) Spectroscopy. CD measurements were performed on a Jasco-720 spectropolarimeter, using a 2-mm cuvette at 20 °C. The peptide concentration was determined by measuring the tyrosine and the tryptophan absorbance in 6 M guanidium chloride, using $\epsilon_{280} = 1300\ M^{-1}\ cm^{-1}$ for Tyr and 5700 for Trp,[14] respectively. The mean residue ellipticity, $[\theta]$, is given in units of deg·cm^2·dmol^{-1}. CD spectra were obtained in 10 mM sodium phosphate buffer (pH 7.0) at a peptide concentration of 20 μM.

Titration of IZ-2W to IZ-2A was carried out in the same buffer by monitoring $[\theta]_{222}$ as a function of the IZ-2W concentration, which was 0, 1, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 10, 15, or 20 μM. The concentration of IZ-2A was 10 μM.

Thermal transition curves were obtained by monitoring $[\theta]_{222}$ as a function of temperature with a 2-mm path length cuvette. The total peptide concentration was 20 μM, and the temperature was increased at a rate of 0.5 °C/min.

Sedimentation Equilibrium Ultracentrifugation. 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 1000 μM in sodium phosphate buffer (10 mM, pH 7.0) containing 100 mM NaCl. 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, using Origin Sedimentation Single Data Set Analysis (Beckman). Partial specific volumes of 0.772 mL/g and 0.771 mL/g were calculated for IZ-2A and IZ-2W, respectively, using the method of Cohn and Edsall.[15]

Size Exclusion Chromatography. IZ-2A, IZ-2W, and a mixture of IZ-2A and IZ-2W (2:1) (20 μM to 1 mM) were dissolved in 0.1 mL of 10 mM sodium phosphate buffer at pH 7.0. The samples were applied to Sephadex G-50 column (0.6 (i.d.) × 9 cm), and were eluted with the same buffer at pH 7.0. One fraction of 90 μL was collected and

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monitored at a wavelength of 230 nm. As the peptide standards, GCN4-pLI[1e], IZ,[6] and GCN4-p1[1e] were used for tetramer, trimer, and dimer, respectively.

Fluorescence Quenching Assay. The fluorescence quenching assay was performed with a HITACHI F-4500 fluorescence spectrophotometer with a 1-cm path-length cuvette. The emission spectra between 280 and 400 nm of tryptophan were measured with excitation at 278 nm. The measurements were performed in 10 mM sodium phosphate and 100 mM NaCl (pH 7.0) at room temperature. The total concentration of peptide was ~20 μM.

Nuclear Magnetic Resonance (NMR). NMR spectroscopy was performed on a Bruker DMX600 spectrometer operated at 600.13 MHz for ^{1}H. Chemical shifts were referenced internally to 0 ppm with trimethylsilylpropionic acid. One-dimensional spectra were measured at 25 °C with suppression of the residual water signal by weak presaturation. The data sets were defined by 8K complex points, and 32 scans were accumulated using a spectral width of 8289.3 Hz. Samples were prepared at an approximate concentration of 1 mM in ^2H_2O (pH 7.0).

The hydrogen-exchange study was initiated by dissolving the lyophilized peptide in ^2H_2O. The concentrations of $(IZ\text{-}2A)_2$/IZ-2W and $(IZ\text{-}2W)_3$ were 0.66 and 0.22 mM, respectively. The signal intensities were normalized against the nonexchangeable ϵ-CH signal (7.18 ppm) of the Tyr residue.

Supporting Information Available: Data of the sedimentation equilibrium analysis of IZ-2W and IZ-2A/IZ-2W (2:1) mixture at pH 7.0. Data of Sephadex G-50 gel filtration and RP-HPLC analyses of IZ-2A/IZ-2W (2:1) mixture. ^{1}H-NOESY, and -COSY spectra of $(IZ\text{-}2A)_2$/IZ-2W complex (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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