Selective Formation of AAB- and ABC-Type Heterotrimeric α-Helical Coiled Coils[+]

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Abstract: The α-helical coiled coils have a representative amino acid sequence of $(abcdefg)_n$ heptad repeats. We previously reported that two peptides named IZ-2A and IZ-2W formed an $(IZ\text{-}2A)_2$/IZ-2W heterotrimer with an Ala–Ala–Trp interaction in the hydrophobic core. In this paper, we describe the selective formation of AAB- and ABC-type heterotrimers. To increase the selectivity of the AAB-type heterotrimeric formation, Lys residues at the *f* position were mutated to either an Ala or a Gln residue to form IZ-2A(*f*A) or IZ-2W(*f*Q). Separately, both IZ-2A(*f*A) and IZ-2W(*f*Q) have a random structure at pH 7 and 20 °C. However, together IZ-2A(*f*A) and IZ-2W(*f*Q) form a 2:1 complex with a thermal transition midpoint (*T*m) of 48 °C. This procedure was applied to prepare the ABC-type heterotrimer, in which two sets of Ala–Ala–Trp interactions were designed in the hydrophobic core. Interhelical interaction between the *e* and *g* positions and the α-helical propensity of the amino acid at the *f* position were also considered in the design. The resultant three peptides selectively formed the ABC-type heterotrimer with a *T*m of 51 °C. Other peptide combinations had random coil properties.

Keywords: coiled coil · de novo design · helical structures · protein design · protein engineering

Introduction

An α-helical coiled coil has a structure in which two to five α-helices wrap around each other.[1] Due to the simple structure and importance of its biological functions, this structure is an attractive target of de novo design.[2] It mediates associations of a large variety of proteins and also induces the homo- or heterooligomerization of proteins. Accordingly, when the domains of proteins or short peptide ligands are fused to α-helical coiled coil structures, multivalent binding molecules are designed and created.[3] Amino- and carboxy-terminal segments of dihydrofolate reductase and green fluorescence protein can be reassembled through the mediation of homo- or heterodimeric coiled coils.[4] Heterooligomeric complexes can be constructed from heterooligomeric coiled coils; hence, these are potential scaffolds for further functional design.

The coiled coil has a representative amino acid sequence of $(abcdefg)_n$ heptad repeats. The *a* and *d* positions are usually occupied by hydrophobic residues and form a hydrophobic core. The amino acids in the hydrophobic core should have more influence on the structure than those at other positions. For example, an Asn amino acid at the hydrophobic core of GCN4 plays an important role in determining the structural specificity.[5] On the other hand, Glu is responsible for the pH-dependent conformational change of the macrophage scavenger receptor.[6] It suggests that if one or two suitable amino acids are properly introduced in the hydrophobic position, then the desired functions might be imparted to the coiled coil. Furthermore, His residues and a Cys residue have been designed in the hydrophobic core of a triple-stranded coiled coil to induce the folding of the peptides by metal ions.[7] Because of the small size of the trimer, and the fact that the *a* and *d* positions are buried deeper

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[+] Abbreviations used in this paper: Rink amide resin, 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin; Hc, 7-hydroxycoumarin; Fmoc, 9-fluorenylmethoxycarbonyl; MBHA, 4-methyl benzhydrylamine resin; *t*Boc, *tert*-butoxycarbonyl; NMP, *N*-methylpyrrolidone; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; CD, circular dichroism; *T*m, transition midpoint.

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than those of the double-stranded coiled coil, the heterotrimer is a target for studies of de novo designed proteins.

We previously prepared a de novo designed peptide, IZ, [YGG(IEKKIEA)$_4$] (*defgabc*), which forms a parallel triple-stranded coiled coil.[8] To construct the AAB-type heterotrimer, we replaced the Ile residue at the *a* position in the second heptad repeat with either an Ala or a Trp residue, to form IZ-2A and IZ-2W, respectively. [9] The IZ-2A/IZ-2W (2:1) heterotrimer formed with a native-like structure. However, IZ-2W also formed the homotrimer, less stable (by 9 °C) than the heterotrimer. In this work, the amino acid at the *f* position was selected to adjust the stability for the selective formation of the AAB-type heterotrimer by destabilizing the homotrimer. We extended this strategy to the ABC-type heterotrimeric coiled coil. In the design, we used the Ala–Ala–Trp interactions two rather than three of the *a* positions, so that one peptide had Ala–Ala, the second peptide had Ala–Trp, and the third peptide had Trp–Ala interactions at the second and third *a* positions of the 32-residue peptides. We also combined ionic interactions at the *e* and *g* positions for further selective formation of the heterotrimer. Finally, the amino acid at the *f* position was chosen for fine tuning the thermal stability.

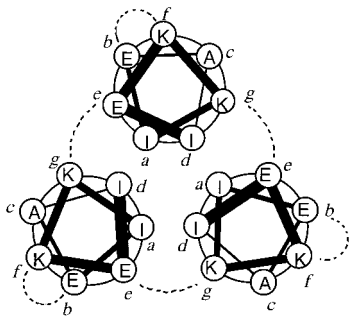

Figure 1. Helical wheel model of the second heptad repeat of IZ. Glu–Lys ion pairs were designed to be formed between the *e* and *g* positions of the neighboring strands. Intrastranded ion pairs were designed to form at the *b* and *f* positions. The ion pairs are indicated by broken lines. For heterotrimer formation, the *a* positions of the three peptides were designed to have a combination of Ala–Ala–Trp interactions. Lys at the *f* positions were changed to either Ala residues or Gln residues. For the ABC-type heterotrimer, in particular, amino acids at the *e* and the *g* positions were also changed to maximize the ionic interactions and minimize the ionic repulsions.

Table 1. Amino acid sequences of the peptides used for heterotrimeric coiled-coils.[a]

		defgabc 1[b]	*defgabc* 2[b]	*defgabc* 3[b]	*defgabc* 4[b]	*defg*
IZ	YGG	IEKKIEA	IEKKIEA	IEKKIEA	IEKKIEA	
IZ-2A	–––	–––––––	––––A––	–––––––	–––––––	
IZ-2W	–––	–––––––	––––W––	–––––––	–––––––	
IZ-2A(*f*A)	–––	––A––––	––A–A––	––A––––	––A––––	
IZ-2W(*b*A)	–––	–––––A–	––––WA–	–––––A–	–––––A–	
IZ-2W(*b*A*f*A)	–––	––A––A–	––A–WA–	––A––A–	––A––A–	
IZ-2W(*f*A)	–––	––A––––	––A–W––	––A––––	––A––––	
IZ-2W(*f*Q)	–––	––Q––––	––Q–W––	––Q––––	––Q––––	
IZ-AA	–––	–––––––	–K––A––	––––A––	–––E–––	IEKG
IZ-AW	–––	–––E–––	–––––A––	–K––W––	–––––––	IEKG
IZ-WA	–––	–––––––	–––EW––	––––A––	–––––––	IKAG
IZ-AW(*f*A)	–––	––AE–––	––A–A––	–KA–W––	––A––––	IEKG
IZ-WA(*f*A)	–––	––A––––	––AEW––	––A–A––	––A––––	IEKG

[a] Only amino acids different from the IZ sequence are indicated. A bar indicates the same amino acid as used in IZ. [b] Heptad number.

Results

Effect of the *b* and *f* positions on the stability of IZ-2W: We previously prepared a de novo designed peptide IZ, [YGG-(IEKKIEA)$_4$] (*defgabc*), which forms a parallel triple-stranded coiled coil (Figure 1, Table 1). [8] The Ile residues at the *a* and *d* positions were used for the hydrophobic packing. Glu–Lys ion pairs between the *e* and *g* positions of the neighboring strands were used to increase the structural stability and to define the parallel orientation of the peptides. We also designed the peptide to have Glu and Lys residues at the *b* and *f* positions, respectively. These charged residues form a salt bridge in the same helix to increase the stability.

To examine the effects of the solvent exposed Glu and Lys residues, we used the IZ-2W peptide, which is a derivative of the IZ peptide and has a Trp residue at the *a* position of the second heptad repeat (Table 1), because it can be easily quantitatively charactiterized, it has moderate thermal stability, and it is a key peptide of the heterotrimeric coiled coil construction. The Glu residues at the *b* position or the Lys residues at the *f* position were substituted with the Ala residues to form IZ-2W(*b*A) and IZ-2W(*f*A), respectively (Table 1). We also prepared IZ-2W(*b*A*f*A), in which the Ala residues occupy both *b* and *f* positions. The structure of the three peptides was analyzed by circular dichroism (CD) spectroscopy. The three peptides exhibited the same α-helical structure in aqueous solution with negative maxima at 208 and 222 nm (Figure 2).

To evaluate the effect of the mutations on the stabilization energy, we analyzed the GdnHCl denaturation of the

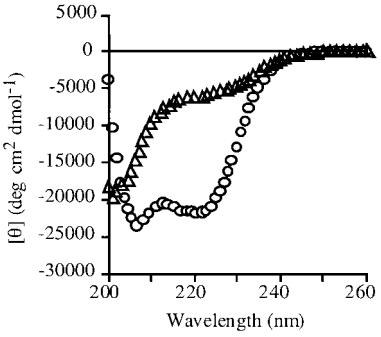

Figure 2. CD spectra of IZ-2W(*f*A). CD spectra were measured in aqueous buffer at pH 7.0 (circles) or in 3 M GdnHCl (triangles) at 20 °C with a total peptide concentration of 20 μM. IZ-2W(*b*A) and IZ-2W(*b*A*f*A) showed the same CD spectra as IZ-2W(*f*A).

peptides by CD spectroscopy. Addition of GdnHCl to solutions of the peptides changed the coiled coil structures to random coils exhibiting a minimum lower than 200 nm with an isosbestic point of 203 nm (Figure 2), showing the two-state model behavior. The $[\theta]$ value at 222 nm was measured as a function of the concentration of GdnHCl at 25 °C and pH 7. As shown in Figure 3, the GdnHCl transition displays

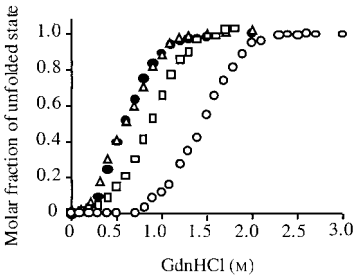

Figure 3. GdnHCl denaturation curves for IZ-2W (closed circles), IZ-2W(*b*A) (squares), IZ-2W(*f*A) (triangles), and IZ-2W(*b*A*f*A) (open circles). The denaturation curves were recorded in 10 mM sodium phosphate at pH 7.0 with a peptide concentration of 20 μM.

cooperative unfolding curves. The free energies of unfolding of the peptides were calculated as described in the Experimental Section. The results are summarized in Table 2 and

Table 2. Physical data for triple-stranded designed coiled-coils.

Peptide	*T*m [°C]	$[GdnHCl]_{1/2}$ [M]	*m* [kcal mol^{-1} mol^{-1}]	$\Delta\Delta G_u$(IZ-2W)[a] [kcal mol^{-1}]
IZ-2W	51	0.62	4.3	0.0
IZ-2W(*b*A)	65	0.91	5.2	+1.4
IZ-2W(*f*A)	43	0.60	5.1	−0.9
IZ-2W(*b*A*f*A)	>80[b]	1.44	3.8	+3.3

[a] A positive value indicates that the analogue is more stable than IZ-2W. [b] Precipitation of IZ-2W(*b*A*f*A) takes place at about 85 °C.

Figure 4. When both Glu residues and Lys residues were changed to Ala residues, the stability of IZ-2W(*b*A*f*A) was increased by 3.3 kcal mol^{-1} in spite of the destruction of the ion pairs between the Glu and Lys residues. When the Glu residues at the *b* position were changed to Ala residues, IZ-2W(*b*A) was also stabilized against the chemical denaturation by 1.4 kcal mol^{-1} in spite of the loss of the ion pairs. On the other hand, stability of IZ-2W(*f*A), in which the Lys residues at the *f* position were changed to Ala residues, decreased by 0.9 kcal mol^{-1}. In consequence, the difference in the structural destabilization by substitution of the Ala with the Lys residue was 1.9 kcal mol^{-1}, and that of the Glu residue was 4.2 kcal mol^{-1} (Figure 4).

The structural stability was also confirmed by the thermal denaturation experiments. Compared with the melting temperature of 51 °C for IZ-2W,[9] those of IZ-2W(*b*A) and IZ-2W(*f*A) were 65 and 43 °C, respectively (Table 2). The most stable peptide, IZ-2W(*b*A*f*A), was stable up to 80 °C. Thus,

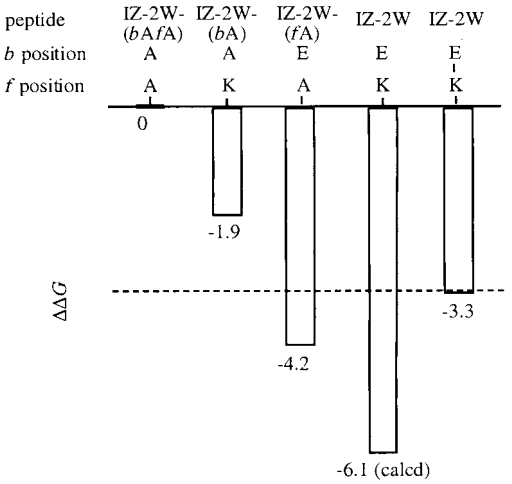

Figure 4. Relationship of $\Delta\Delta G$ [kcal mol^{-1}] values of IZ derivatives. This figure depicts the ΔG value of IZ-2W(*b*A*f*A) as the standard to clarify the effect of the substitution of the *b* and *f* positions. The broken line shows the ΔG value of IZ-2W as the standard, which corresponds to that given in Table 2. Amino acids at the *b* and *f* positions are indicated below the peptide name. A bar between the amino acids indicates the ion pair between the Glu and the Lys residues. The $\Delta\Delta G$ value (−6.1 kcal mol^{-1}) of IZ-2W without the ion pair was calculated by adding the $\Delta\Delta G$ values of IZ-2W(*b*A*f*A) and IZ-2W(*f*A). Contribution of the ion pairs to the stability is calculated by the difference in $\Delta\Delta G$ values of IZ-2W and IZ-2W without the ion pair. A negative value means a decrease in stability.

the thermal stability was correlated with the stability against chemical denaturation.

AAB-type heterotrimer: When we changed the amino acids at the *b* and *f* positions of IZ-2W to Ala residues, the substitution at the *f* position had more influence relative to that at the *b* position on the stability of IZ-2W. The IZ-2W(*f*A) peptide still formed a homotrimer (Figures 2 and 3). A Gln residue has lower α-helical propensity than an Ala residue,[10] and the Gln residue at the *f* position destroys the Glu–Lys ion pair, as in the case of the Ala residue. Substitution with the Gln residue is expected, therefore to provide a greater destabilization effect on the homotrimer formation than the Ala residue. Actually, IZ-2W(*f*Q) exhibited a random structure in the CD spectrum with minimum below 200 nm (Figure 5a). IZ-2A exhibited a weak tendency towards formation of an α-helical structure.[9] To destabilize the α-helical structure further, the *f* position of the IZ-2A was substituted with the Ala residue to form IZ-2A(*f*A). IZ-2A(*f*A) exhibited the random structure, as observed for IZ-2W(*f*Q), in the CD spectra (Figure 5a).

A 2:1 mixture of IZ-2A(*f*A) and IZ-2W(*f*Q) with total peptide concentrations of 20 μM at pH 7 exhibited a helical conformation that differs from the case of the single peptides (Figure 5a), suggesting that the two peptides formed a complex. The CD spectrum showed a $[\theta]_{222}/[\theta]_{208}$ ratio of less than 1, suggesting that the complex fluctuates at the end of the coiled coil or α-helical bundle structure.[11] Thus, in the case of IZ-2A(*f*A) and IZ-2W(*f*Q), the peptides formed a complex with a highly α-helical structure, while each peptide separately showed a random structure. Then, thermal

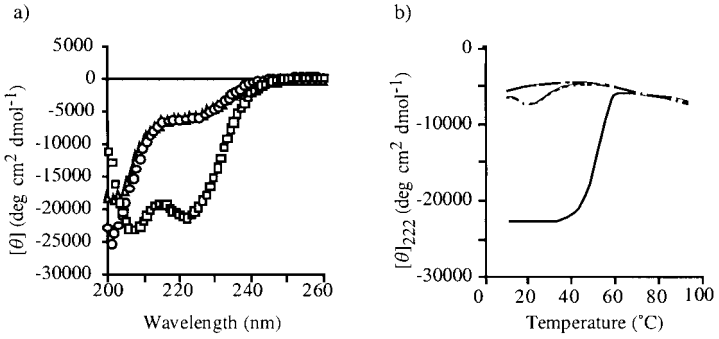

Figure 5. a) CD spectra of IZ-2A(*f*A) (triangles), IZ-2W(*f*Q) (circles), and IZ-2A(*f*A)/IZ-2W(*f*Q) (2:1) mixture (squares). b) Thermal denaturation curves of IZ-2A(*f*A) (dash-dotted line), IZ-2W(*f*Q) (broken line), and the IZ-2A(*f*A)/IZ-2W(*f*Q) (2:1) mixture (solid line). The CD spectra and the thermal denaturation were measured at 20 °C and pH 7.0 with a total peptide concentration of 20 μM.

denaturation was carried out to assess the stability and the cooperativity of their association. The thermal denaturation curves are shown in Figure 5b. The mixture of IZ-2A(*f*A)/IZ-2W(*f*Q) (2:1) showed a sharp melting transition with a *T*m of 48 °C. To assess the stoichiometry of the IZ-2A(*f*A)/IZ-2W(*f*Q) complex, the $[\theta]_{222}$ value of IZ-2A(*f*A) was monitored as a function of concentration of IZ-2W(*f*Q) (Figure 6). The $-[\theta]_{222}$ value increased up to the IZ-2A(*f*A)/IZ-2W(*f*Q) ratio of 2, and then decreased. This result indicates that IZ-2A(*f*A) and IZ-2W(*f*Q) interact in a 2:1 ratio.

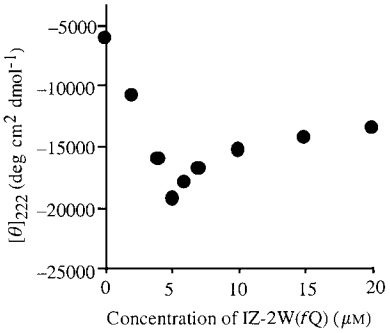

Figure 6. IZ-2W(*f*Q) titration profile of IZ-2A(*f*A) monitored by CD spectroscopy at 20 °C and pH 7.0. The $[\theta]_{222}$ was monitored and plotted as a function of the concentration of IZ-2W(*f*Q). The IZ-2A(*f*A) concentration was 10 μM.

The peptide oligomerization was determined by sedimentation equilibrium centrifugation analysis (Figure 7a). A mixture of IZ-2A(*f*A)/IZ-2W(*f*Q) (2:1) with a peptide concentration range of 20–100 μM gave an apparent molecular mass of 10050 ± 299 Da, indicating that the peptides were trimerized (the calculated molecular mass for (IZ-2A(*f*A))$_2$/IZ-2W(*f*Q) is 10159 Da). Moreover, when analyzed by Sephadex G-50 gel filtration chromatography (Figure 7b), the complex was eluted at the fraction corresponding to a trimerized peptide.

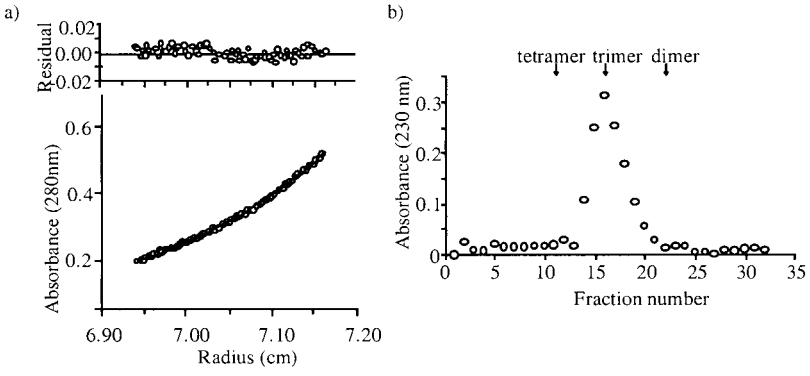

Figure 7. a) Sedimentation equilibrium analyses of the mixture of IZ-2A(*f*A) and IZ-2W(*f*Q) (2:1). See Experimental Section for details of the measurements. The apparent molecular mass was 10349 Da (calculated molecular mass 10159 Da). When a peptide concentration of 20 μM was used, the apparent molecular mass was 9751 Da. b) Gel filtration analysis of a mixture of IZ-2A(*f*A) and IZ-2W(*f*Q) (2:1; 20 μM). See Experimental Section for details of the measurements..

ABC-type heterotrimer: Based on the IZ peptide, we designed three peptides for the selective construction of the ABC-type heterotrimer (Table 1). The *a* positions of the second and third heptad were occupied by a combination of either Ala–Ala, Trp–Ala, or Ala–Trp interactions (Table 1). To choose the best suited combination of the amino acid at the *e* and *g* positions as described by Nautiyal et al. for the heterotrimerization, [12] one amino acid at both positions was changed from Glu to Lys or from Lys to Glu in each peptide. Four amino acids were extended to the C-terminus to increase the number of ion pairs formed to stabilize the heterotrimer. We characterized the three peptides as IZ-AA, IZ-AW, and IZ-WA, from the order of appearance of the Ala and Trp residues.

The CD spectra of the three separate peptides exhibited random structures with a minimum below 200 nm (Figure 8a). Mixtures of two of the peptides also exhibited random structures, except for a mixture of IZ-AW and IZ-WA, which showed a weak α-helical structure as indicated by the minimum at 222 nm. On the other hand, an equimolar mixture of the three peptides exhibited a highly α-helical structure with negative maxima at 208 and 222 nm. This result indicates an assembly of the three peptides to form an α-helical structure.

To destabilize the complex from IZ-AW and IZ-WA, the amino acids at the *f* positions of the two peptides were changed to Ala residues to form IZ-AW(*f*A) and IZ-WA(*f*A), respectively (Table 1). By this substitution, a mixture of IZ-AW(*f*A) and IZ-WA(*f*A) exhibited the random structure, while an equimolar mixture of the three peptides still showed the α-helical structure (Figure 8b). When we measured thermal denaturation of an equimolar mixture of the three peptides, a steep transition curve was observed with a *T*m of 51 °C, showing a highly cooperative transition (Figure 8c). The peptide oligomerization was determined by sedimentation equilibrium centrifugation analysis (Fig-

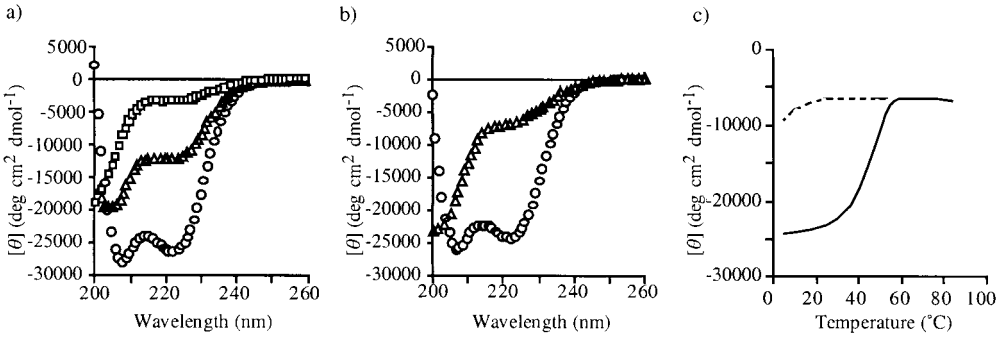

Figure 8. a) CD spectra of IZ-AA (squares) and a mixture of IZ-AW/IZ-WA (1:1) (triangles), and a mixture of IZ-AA/IZ-AW/IZ-WA (1:1:1) (circles). IZ-WA, IZ-AW, a mixture of IZ-AA/IZ-WA (1:1), and a mixture of IZ-AA/IZ-AW (1:1) exhibited the same CD spectra as IZ-AA. b) CD spectra of a mixture of IZ-AA/IZ-WA(*f*A)/IZ-AW(*f*A) (1:1:1) (circles), and a mixture of IZ-AW(*f*A)/IZ-WA(*f*A) (1:1) (triangles). c) Thermal denaturation curves of a mixture of IZ-AA/IZ-WA(*f*A)/IZ-AW(*f*A)(1:1:1) (solid line) and a mixture of IZ-WA(*f*A)/IZ-AW(*f*A)(1:1) (broken line). The CD spectra and the thermal denaturation were measured at 20 °C and pH 7.0 with a total peptide concentration of 20 μM.

ure 9a). The mixture of three peptides at a peptide concentration range of 20–100 μM gave an apparent molecular mass of 11309 ± 313 Da (calculated molecular mass 11316 Da) indicating the trimerization of the peptides. Moreover, we

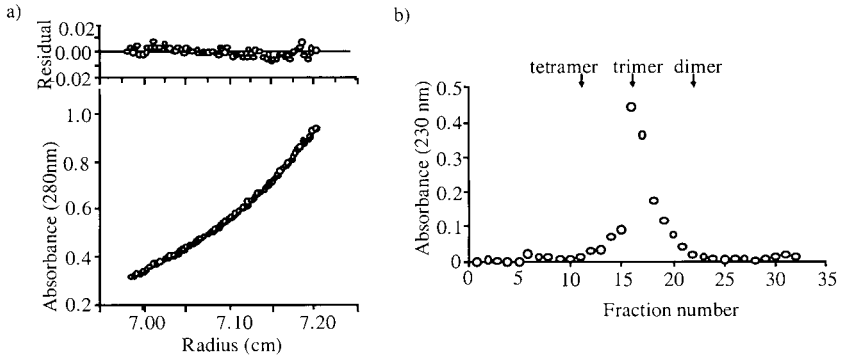

Figure 9. a) Sedimentation equilibrium analyses of the mixture of IZ-AA, IZ-AW(*f*A) and IZ-WA(*f*A) (1:1:1). See Experimental Section for details of the measurements. The apparent molecular mass was 11622 Da (calculated molecular mass 11316 Da). When a peptide concentration of 20 μM was used, the apparent molecular mass was 10996 Da. b) Gel filtration analysis of a mixture of IZ-AA, IZ-AW(*f*A), and IZ-WA(*f*A) (1:1:1; 20 μM). See Experimental Section for details of the measurements.

confirmed the trimerized complex by Sephadex G-50 gel filtration chromatography (Figure 9b).

Fluorescence of the Trp residue is sensitive to the environment of the indole side chain. A Trp residue shows fluorescence emission maxima at 327–332 nm in a hydrophobic environment and 354 nm in water.[13] The fluorescence of the Trp residues in the complex, IZ-AA/IZ-AW(*f*A)/IZ-WA(*f*A), was measured to identify the environment of the indole side chain of the Trp residue. The fluorescence maximum of the Trp residues was at 333 nm after excitation at 278 nm (data not shown). This result showed that the Trp residues are completely buried in the hydrophobic core of the complex as in the case of $(IZ\text{-}2A)_2$/IZ-2W.[9]

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 heterotrimer, a fluorescent probe, 7-hydroxycoumarin (Hc), was coupled to the N-termini of IZ-AA, IZ-AW(*f*A), and IZ-WA(*f*A). 7-Hydroxycoumarin is known to have less effect on the aggregation degree and stabilization of the coiled coil structure.[14] As shown in Figure 10, when the N-terminal 7-hydroxycoumarin-tagged peptides, Hc-IZ-AA, Hc-IZ-AW(*f*A), and Hc-IZ-WA(*f*A) were combined, the fluorescence was quenched to below 20% of the original fluorescence intensity. On the other hand, when a C-terminal-tagged peptide, IZ-AA-Hc, was mixed with two N-terminal-tagged peptides, Hc-IZ-AW(*f*A) and Hc-IZ-WA(*f*A), the fluorescence was quenched by only 30%. These results indicate that the three peptides have a parallel orientation in the bundle structure.

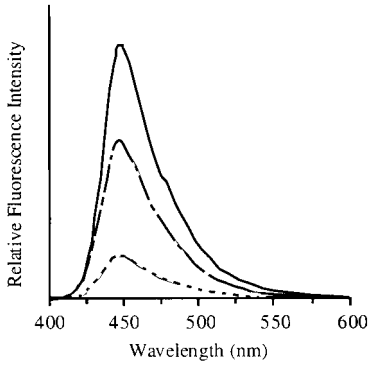

Figure 10. Fluorescence spectra of Hc-IZ-AA (solid line), a mixture of Hc-IZ-AA/Hc-IZ-WA(*f*A)/Hc-IZ-AW(*f*A) (1:1:1) (broken line) and a mixture of IZ-AA-Hc/Hc-IZ-WA(*f*A)/Hc-IZ-AW(*f*A) (1:1:1) (dash-dotted line). The spectra were recorded in 10 mM sodium phosphate, pH 7.0 and 100 mM NaCl with a total peptide concentration of 20 μM.

Discussion

The AAB-type heterotrimer was constructed by manipulation of single Ala and Trp residues at the hydrophobic *a* position of the designed triple-stranded coiled coil.[9] Although substitution by an Ala residue at the hydrophobic position largely destabilized the α-helical formation, substitution by a Trp residue minimally affected the stabilization of the homotrimeric structure.[9] We tried to destabilize the homotrimer of the peptide that contained the Trp residue (IZ-2W) without affecting the stability of the heterotrimer.

Considering the three peptides, IZ-2W(*b*A*f*A), IZ-2W(*b*A), and IZ-2W(*f*A), the first two peptides were 3.3 and 1.4 kcal mol^{-1} more stable, respectively, than the parent peptide, IZ-2W. On the other hand, the third peptide was 0.9 kcal mol^{-1} less stable; this shows that substitution at the *f* position destabilizes the homotrimer (Figure 4). The decrease of the stabilization energy by substitution of an Ala residue was 1.9 and 4.2 kcal mol^{-1} for the Lys and Glu residues, respectively, relative to that of the Ala residue. Each peptide has twelve *b* or *f* positions in the trimer form. Therefore, the contribution of one substitution was calculated to be 0.16 and 0.35 kcal mol^{-1} for the Lys and Glu residues, respectively. This result shows that the Lys residue has higher α-helical propensity than the Glu residue in the case of the triple-stranded coiled-coil, although a contribution of factors other than the intrinsic helical propensity might be involved in the enhanced stability. In the α-helix, the Glu residue has similar α-helical propensity to that of the Lys residue.[15] On the other hand, stabilization energies of the Ala, Lys, and Glu residues at the *f* position in the double-stranded coiled coil were reported as −0.77, −0.65, and −0.27 kcal mol^{-1}, respectively, relative to Gly.[10] Accordingly, the Ala residue stabilizes the α-helical structure by 0.12 kcal mol^{-1} more than the Lys residue, and by 0.42 kcal mol^{-1} more than the Glu residue. Thus, similar results were obtained in the coiled coil with different amino acid sequences.

If the Glu residue and the Lys residue do not form ion pairs, IZ-2W should be destabilized with respect to IZ-2W(*b*A*f*A) by 6.1 kcal mol^{-1} (1.9+4.2 kcal mol^{-1}; Figure 4). Since the difference in stabilization energy between IZ-2W and IZ-2W(*b*A*f*A) was found to be 3.3 kcal mol^{-1}, the stabilization energy of the ion pairs was calculated to be 2.8 kcal mol^{-1}. The ion pair between the *i* and *i*+4 positions is preferable to that between the *i* and *i*+3 positions.[16] In the triple-stranded coiled coil, therefore, there are nine ion-pair interactions. In consequence, the ion-pair stabilization energy of Glu and Lys at the *i* and *i*+4 positions was calculated to be 0.31 kcal mol^{-1}. The stabilization by the intrahelical Glu–Lys ion pair at the *i* and *i*+4 positions is 0.38–0.5 kcal mol^{-1}.[11b,15b,17] These values are consistent with our results.

The selective construction of the AAB-type heterotrimeric coiled coil was successfully accomplished by manipulation of amino acid at the *f* position (Figure 5 and Table 1). Nautiyal et al.[12a] and Kiyokawa et al.[18] previously designed an ABC-type heterotrimeric coiled coil by different design strategies, employing ionic interactions at the *e* and *g* positions or Ala–Ala–Trp interactions in the hydrophobic core to mediate heterospecificity. Schnarr and Kennan tried to use an unnatural amino acid to construct the ABC-type heterotrimer.[19] Among all the peptide combinations, the ABC-type heterotrimer was the most stable in those cases; however, other alternative species composed of one or two peptides also form the triple-stranded coiled coil structures. Here, in our design, as well as ionic interactions between the *e* and *g* positions, combinations of two Ala residues and one Trp residue are placed in the hydrophobic positions. Moreover, by choosing the amino acid at the *f* position to adjust the stability of the homo- and heterotrimer, we were able to attain the selective construction of the ABC-type heterotrimer. No other species composed of other peptide combinations formed the α-helical structure. Furthermore, we used only natural amino acids for the AAB- or ABC-type heterotrimeric coiled coil. The coiled coil reported here, therefore, can be directly applied in vivo as well as in vitro.

Experimental Section

Peptide synthesis and purification: Peptides were synthesized on an Applied Biosystems Model 433A automated synthesizer, by using Rink amide resin, (substitution 0.37 mmol g^{-1}), based on the standard Fastmoc 0.1 mmol protocol. Attachment of 7-hydroxycoumarin to the N-terminus of the peptides was carried out according to the published procedure,[14] after elongation with two Gly residues as a spacer. Attachment of 7-hydroxycoumarin to the C-terminus was carried out as follows. *N*-α-*t*Boc-*N*-ε-Fmoc-lysine was coupled to the MBHA resin, and then treated with 20% piperidine in NMP for 20 min to remove the Fmoc group. 7-Hydroxycoumarin was then coupled to the lysine by means of the same procedure. The *t*Boc group was removed by treatment 50% TFA/CH_2Cl_2 for 15 min followed by neutralization with 5% diisopropylethylamine in NMP. After coupling with Fmoc-Gly-OH, subsequent peptide elongation was carried out on the automated synthesizer without a capping step. The peptide, prepared on the Rink amide resin, was simultaneously cleaved/deprotected with TFA/water (95:5 v/v) for the peptides without Trp, or TFA/1,2-ethandithiol/water (95:2.5:2.5 v/v) for the other peptides, for 1.5 h. A peptide synthesized on MBHA resin was treated with a mixture of TFA, *m*-cresol and trifluoromethanesulfonic acid (9:1:1, v/v) for 1 h. Deprotected peptides were purified by reversed-phase HPLC. Purification was carried out on a YMC-Pack ODS-A column (10 mm i.d. × 250 mm, 5 μm, YMC, Japan) with a linear gradient of 30 to 50% CH_3CN/H_2O containing 0.1% TFA over the course of 30 min. The peptides were eluted between 20–27 min. The final product was characterized by analytical HPLC and was confirmed by MALDI-TOF mass spectrometry. MS: *m*/*z* calcd for IZ-2W(*b*A): 3383; found: 3385; MS: *m*/*z* calcd for IZ-2W(*f*A): 3387 found: 3389; MS: *m*/*z* calcd for IZ-2W(*b*A*f*A): 3155; found: 3156; MS: *m*/*z* calcd for IZ-2A(*f*A): 3272; found: 3271; MS: *m*/*z* calcd for IZ-2W(*f*Q): 3615; found: 3616; MS: *m*/*z* calcd for IZ-AA: 3886; found: 3887;; MS: *m*/*z* calcd for IZ-AW: 4001; found: 4000; MS: *m*/*z* calcd for IZ-WA: 4001; found: 4001; MS: *m*/*z* calcd for IZ-AW(*f*A): 3715; found: 3715; MS: *m*/*z* calcd for IZ-WA(*f*A): 3715; found: 3716; MS: *m*/*z* calcd for Hc-IZ-AA: 3908; found: 3910; MS: *m*/*z* calcd for Hc-IZ-AW(*f*A): 3739; found: 3739; MS: *m*/*z* calcd for Hc-IZ-WA(*f*A): 3739; found: 3740; MS: *m*/*z* calcd for IZ-AA-Hc: 4093; found: 4095.

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, with ε_{280} = 1300 and 5700 M^{-1} cm^{-1} for Tyr and Trp, respectively.[20] The mean residue molar ellipticity, [θ], is given in units of deg cm^2 dmol^{-1}. CD spectra were obtained in 10 mM sodium phosphate and 100 mM NaCl buffer (pH 7.0) at a peptide concentration of 20 μM.

Titration of IZ-2W(*f*Q) with IZ-2A(*f*A) was carried out in the same buffer by monitoring $[\theta]_{222}$ as a function of the IZ-2W(*f*Q) concentration, which was 0, 2, 4, 5, 6, 7, 10, 15, or 20 μM. The concentration of IZ-2A(*f*A) 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 $1\,^{\circ}C\,min^{-1}$.

The GdnHCl denaturation curve, which was obtained by monitoring $[\theta]_{222}$ by averaging ten 1.0 s readings, was obtained with a 20 μM peptide solution in 10 mM sodium phosphate and 100 mM NaCl buffer (pH 7.0) at 25 °C. The free energy of unfolding (ΔG) at a given concentration of GdnHCl was calculated from Equation (1):

$$\Delta G = -RT\ln K \quad (1)$$

in which R is the gas constant, T is the absolute temperature, and K is the equilibrium constant for the unfolding process. For a two-state equilibrium between the monomer and the trimer, K was obtained from Equation (2):

$$K = (3c^2 f_u^3)/(1-f_u) \quad (2)$$

in which c is the total peptide concentration and f_u is the fraction of the unfolded (monomeric) peptide. The free energy of unfolding in water (ΔG°) was estimated from a linear extrapolation, according to Equation (3):

$$\Delta G = \Delta G^{o} - m[\mathrm{GdnHCl}] \quad (3)$$

in which m is the average slope of the denaturation curves.[13] To determine the difference in the free energy of unfolding of peptide analogues, $\Delta\Delta Gu$ of the analogues relative to IZ-2W were accurately obtained using Equation (4):

$$\Delta\Delta Gu = \{([\mathrm{GdnHCl}]_{1/2})_{\mathrm{peptide}} - ([\mathrm{GdnHCl}]_{1/2})_{\mathrm{IZ\text{-}2W}}\}(m_{\mathrm{IZ\text{-}2W}} + m_{\mathrm{peptide}})/2 \quad (4)$$

in which $[\mathrm{GdnHCl}]_{1/2}$ is the GdnHCl concentration at the transition midpoint.[21]

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 and 100 μM in sodium phosphate buffer (10 mM, pH 7.0) containing 100 mM NaCl. The samples were rotated at 25 000 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, by using Origin Sedimentation Single Data Set Analysis (Beckman). Partial specific volumes of 0.769, 0.759, 0.758, 0.747, 0.765, 0.752, and $0.752\,mL\,g^{-1}$ were calculated at 20 °C for IZ-2A, IZ-2A(*f*A), IZ-2W(*f*A), IZ-2W(*f*Q), IZ-AA, IZ-AW(*f*A), and IZ-WA(*f*A), respectively, by using the method of Cohn and Edsall.[22]

Size exclusion chromatography: A mixture of IZ-2A(*f*A) and IZ-2W(*f*Q) (2:1) and a mixture of IZ-AA, IZ-AW(*f*A), and IZ-2W (1:1:1) (20 μM–1 mM) were dissolved in sodium phosphate buffer (10 mM, 0.1 mL, pH 7.0). The samples were applied to a Sephadex G-50 column (0.6 (i.d.) × 9 cm), and eluted with the same buffer at pH 7.0. Fractions containing 90 μL were collected and monitored at a wavelength of 230 nm. As for the peptide standards, GCN4-pLI, [2c] IZ,[8] and GCN4-p1[2c] were used for the 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 of Trp between 280–400 nm were measured with excitation at 278 nm. The emission spectra between 400–600 nm of 7-hydroxycoumarin were measured with excitation at 386 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 about 20 μM.

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