*Helical Structures*

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# High Thermal Stability Imparted by a Designed Tandem Arg–Trp Stretch in an α-Helical Coiled Coil**

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Studies on de novo designed proteins involve the construction of unique tertiary structures and the creation of novel functions. Protein structures are mainly formed by various interactions, such as ion-pair, hydrogen-bonding, and hydrophobic interactions. Recently, the cation–π interaction was noted as one of the effective interactions judging from various protein structures in the PDB (protein data bank) databases,[1] and it is thought to contribute to protein structure stabilization and protein–ligand interactions.[2–4] The cation–π interaction is formed between aromatic rings and cationic groups.[1b,5] In proteins, Trp, Tyr, Phe, and His are aromatic residues, while Lys and Arg are cationic residues, and the interaction between Trp and Arg is considered to be the best combination.[1c,6] The cation–π interaction is favored several-

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fold over the formation of a salt bridge in water, in terms of the desolvation energy penalty of the ionic residues. A cation–π interaction stabilizes proteins by 2–3 kcal mol$^{-1}$ more than an ion-pair interaction, which is estimated to contribute 1–3 kcal mol$^{-1}$.[7] Thus, the cation–π interaction provides strong and specific interactions, and therefore it should be considered in protein design.

Designed cation–π interactions have been assessed using the solvent-exposed sites of the short α helices, turn structure, and coiled-coil structure.[8] These data suggested that the contributions of the cation–π interactions to the stability depend on the species and the positions of aromatic and cationic amino acids. However, the effects of the cation–π interactions on the structural stability are quantitatively lower than the values obtained from calculations, probably as a result of the flexibility of the amino acid side chains on the freely exposed sites of the α helix.

The location of the cation–π interaction is neither completely solvent-exposed nor deeply buried within the protein.[6] It is often found between a solvent-exposed site and a completely buried site. We designed a cation–π interaction to reside at the interface between two α helices of a coiled-coil structure. The coiled coil consists of several α helices wrapped around each other, and it represents a heptad repeat sequence designated by *abcdefg* from each position.[9] The *e* and *g*′ positions exist between the hydrophobic–hydrophilic interfaces and are close to each other between two adjacent α helices. Glu and Lys at these positions are close and form an ion pair, which contributes to the stabilization and configuration of the coiled-coil structure. Therefore, the *e* and *g*′ positions of the coiled coil might be suitable places to design a cation–π interaction. We designed a cation–π interaction at the *e* and *g*′ positions and compared the ion-pair interaction between Glu and Lys at the same positions. Our results support the existence of the cation–π interaction.

A de novo designed peptide (IZ), [YGG(IEKKIEA)$_4$] (*defgabc*), forms a parallel triple-stranded α-helical coiled-coil structure.[10] The IZ peptide has a melting temperature ($T_m$) of more than 95 °C, which makes it difficult to compare the thermal stabilities of coiled-coil structures. Therefore, the amino acid at the *a* position of the second heptad was substituted with Gln to adjust the stability and to increase the uniqueness of the structure.[11] We also mutated the amino acid residues on the IZ derivatives, where Lys at the *g* position and Glu at the *e* position were reversed. The prepared peptides are listed in Table 1. There are three possible sites for interactions using the *e* and *g*′ positions after homotrimerization. Trp and Arg were placed in the middle of the peptide. The rest of the *e* and *g*′ positions contained Glu and Lys, respectively, for the ion-pair interactions. A series of cation–π interactions was designed, and the peptides containing Trp and Arg were named WR1, WR2, and WR3, depending on the number of Trp–Arg sets. The *e* position of one peptide and the *g*′ position of the other peptide were alternated between the two α helices, and therefore Trp and Arg were designed to generate an extended array of cation–π interactions, formed by two and three Trp–Arg sets for WR2 and WR3, respectively. WRRW has two Trp–Arg sets; however, because of the repulsive positive charge from Arg, the two Trp–Arg interactions should work separately.

Molecular modeling of WR3 was performed by using molecular mechanics calculations to obtain the preferable spatial position of Trp and Arg residues in the α-helical coiled-coil structures.[12] The energy-minimized WR3 model suggested that the designed three Trp–Arg sets were placed close enough together for cation–π interactions (Figure 1).

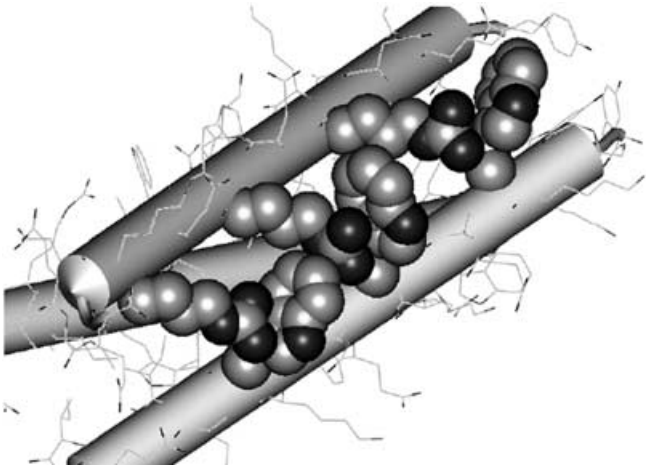

***Figure 1.*** Energy-minimized model of WR3, which was constructed on the basis of a leucine-zipper coiled-coil structure (PDB ID: 1GCM) as template, followed by energy minimization with the OPLS-AA force field and GB/SA solvation model. Trp and Arg residues are illustrated by space-filling molecular graphics.

Circular dichroism (CD) spectroscopy was performed to characterize the secondary structures of the designed peptides (Figure 2). All of the peptides exhibited the spectra of typical α-helical structures, with the characteristic minima at 208 and 222 nm. To analyze the stoichiometry of the peptide aggregates, they were subjected to gel filtration on Sephadex G-50. The peptides eluted at the same position as the standard triple-stranded coiled-coil peptide with a similar amino acid length. These results indicate that the peptides assembled into the trimer with a coiled-coil structure.

The thermal stabilities of the peptides were analyzed by monitoring $[\theta]_{222}$ as a function of temper-

***Table 1:*** Amino acid sequences of the designed coiled-coil peptides and their $T_m$, $\Delta T_m$ [°C], and $\Delta G$ [kcal mol$^{-1}$] values after homotrimerization.

| Peptide | Sequence | | | | | $T_m$ | $\Delta T_m$ | $\Delta G$ |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | | | |
| EK | YGG | IKKEIEA | IKKEQEA | IKKEIEA | IKKEIEA | 52 | 0 | −15.3 |
| WR1 | -- | --- | --W-- | -R-- | --- | 58 | 6 | −15.8 |
| WR2 | -- | --W-- | -R-W-- | -R-- | --- | 75 | 23 | −16.7 |
| WR3 | -- | --W-- | -R-W-- | -R-W-- | -R-- | >95 | n.d. | n.d. |
| WRRW | -- | --W-- | -R-R-- | -W-- | --- | 65 | – | – |
| AA | -- | --- | --A-- | -A-- | --- | 42 | – | −14.6 |
| WA | -- | --- | --W-- | -A-- | --- | 48 | – | −14.8 |
| AR | -- | --- | --A-- | -R-- | --- | 45 | – | −14.7 |


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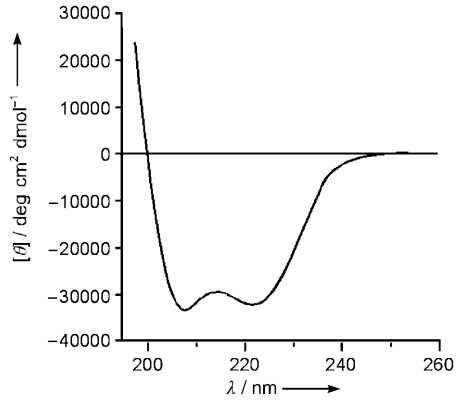


***Figure 2.*** CD spectrum of WR3. EK, WR1, WR2, and WRRW exhibited the same CD spectra as WR3. The measurement was performed in sodium phosphate (20 mM, pH 7.0) containing NaCl (0.1 M) at 20 °C. The peptide concentration was 20 μM.

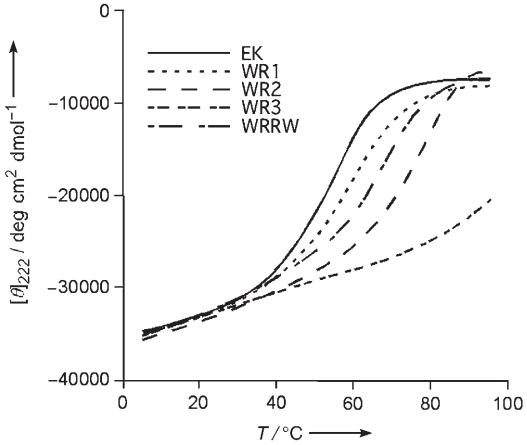


***Figure 3.*** Thermal denaturation curves for EK, WR1, WR2, WR3, and WRRW. The curves were recorded in sodium phosphate (20 mM, pH 7.0) containing NaCl (0.1 M), with a peptide concentration of 20 μM.

ature (Figure 3). EK exhibited a $T_m$ of 52 °C. When one Glu–Lys set was changed to Trp–Arg, the $T_m$ was increased to 58 °C (WR1), which is 6 K higher than that of EK, in spite of the removal of one of the ion pairs. This finding implies the presence of an interaction between Trp and Arg which is stronger than that of the ion pair. The effect on the thermal stability was more remarkable when a couple of Trp–Arg sets were placed at the *e* and *g* positions. WR2 exhibited a $T_m$ of 75 °C, which is 23 K higher than that of EK. On the other hand, WRRW had a $T_m$ of 65 °C, which is 13 K higher than that of EK. This 13-K increase is almost twice the $\Delta T_m$ between EK and WR1, which indicates that the two sets of Trp–Arg in WRRW do not interact cooperatively but separately, and that the orientation of WR (N→C or C→N) does not affect the stability of the coiled-coil structure. WR2 has an even higher $T_m$ value, which is 10 Kmore than that of WRRW. Furthermore, when three Trp–Arg sets were used, the α-helical conformation was significantly more stable, leading to a $T_m$ value greater than 95 °C for WR3. These results suggest that when Trp and Arg are aligned contiguously between two α helices, they interact cooperatively. It is apparent that the alternating alignment of aromatic and cationic side chains multiplies their effects on the thermal stability.

The fluorescence maximum of Trp is 327–332 nm under hydrophobic conditions and 354 nm in solvent-exposed sites.[13] We placed Trp at the *g* position, at the edge of the hydrophobic and hydrophilic environments. Trp might participate in the hydrophobic interaction, rather than the interaction with Arg, because the aromatic residues tend to be buried in the proteins. Therefore, we measured the fluorescence spectrum of the Trp residue. All of the peptides exhibited fluorescence maxima at 347–351 nm, with their intensities depending on the number of Trp residues. This finding indicates that the Trp residues exist at the solvent-exposed sites, and do not contribute to the hydrophobic interactions. The Trp residues in both WR2 and WRRW reside at solvent-exposed sites; however, WR2 is thermally more stable than WRRW. This result also supports the conclusion that the contiguous alignment of Trp and Arg augments the structural stability.

To obtain further physicochemical information concerning the Trp–Arg interaction, we prepared three more derivative peptides, AA, WA, and AR (see Table 1). These peptides formed triple-stranded coiled-coil structures. We carried out Gdn·HCl (guanidine hydrochloride) denaturation experiments to calculate the stabilization energy $\Delta G$. The $\Delta G$ values obtained for AA, WA, and AR were similar at −14.6, −14.8, and −14.7 kcal mol$^{-1}$, respectively. Thus, the side chain of Trp or Arg alone does not contribute to the structural stability. The fact that the stabilization energies of EK and WR1 were −15.3 and −15.8 kcal mol$^{-1}$, respectively, indicates that an interhelical interaction between Glu and Lys, or Trp and Arg, was formed, and that one Trp–Arg interaction is stronger than one Glu-Lys ion-pair interaction by 0.17 kcal mol$^{-1}$. WR2 is even more stable, with a $\Delta G$ value of −16.7 kcal mol$^{-1}$, which shows that the stabilization energy of one Trp–Arg interaction is more than 0.23 kcal mol$^{-1}$ higher than that of one Glu–Lys ion pair. Thus, one continuous Trp–Arg interaction stabilizes the structure even more, by 0.06 kcal mol$^{-1}$.

In conclusion, we have designed a cation–π interaction at the interface between two α helices. Physicochemical experiments suggested the existence of an interaction between Trp and Arg, and showed that its contribution to stabilizing the protein structure is greater than that of an ion pair. In particular, the tandem repeats of Trp and Arg alignment, which imply a network of cation–π interactions, substantially stabilize the structure. The extended cation–π interaction is observed in natural proteins, such as the human growth hormone receptor extracellular domain.[1a,14] Although the role of the interaction was not revealed by the amino acid mutation, it may function to increase the protein stability. Our design strategy using the Trp–Arg interaction should contribute to increase protein stability and specificity.

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