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Selective Protein−Protein Interactions Driven by a Phenylalanine Interface

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Abstract: Highly specific protein−protein interfaces have been the subject of considerable study for their potential utility in disrupting or interrogating cellular signaling and control networks. We report that coiled-coil sequences decorated with phenylalanine core residues fold into stable α-helical bundles and that these self-sort from similar peptide assemblies with aliphatic core side chains. For self-assembled ensembles derived from 30-residue monomeric peptides, the ΔG of specificity is −1.5 kcal/mol, comparable with earlier self-sorting coiled-coil systems. Intriguingly, although this interface is constructed from canonical amino acids, it does not appear to have been exploited in native proteins.

Introduction

Proteins often are part of complex networks that are sensitive to the environment and respond actively to external stimuli.[1] The attendant biological functions of protein complexes are largely dependent on the selectivity with which they bind their partners under given conditions.[2,3] The ability to program specific, orthogonal oligomerization of proteins is highly useful in such contexts. Coiled-coils are valuable precisely because they can be programmed to partner (e.g., in homo- or heteromeric sense, or with different oligomerization states) according to well-understood sequence rules.[4] This versatility, combined with their synthetic accessibility, has allowed their incorporation into many interesting self-assembled systems.[5] Coiled-coils have proven especially useful in the construction of dynamic[6,7] and large-scale[8] aggregate structures.

Variably interacting coiled-coil domains also form the basis for both a molecular network of eukaryotic transcription factors[3] and a recently reported artificial peptide network.[7] Such networks offer an avenue to understand the detailed molecular mechanisms of cellular information processing and stimulus response.[1] Thus, in addition to extending the toolkit available for peptide-based self-assembly, orthogonal protein−protein interfaces may be useful tools for delineating[9] or augmenting[10] natural and artificial protein networks.

The rigid, expansive hydrophobic surfaces of aromatic amino acids have proved useful for protein design, in particular allowing the 20-residue Trp-cage miniprotein to adopt a stable, folded conformation.[11] Aromatic amino acids are also represented in helical oligomerization domains of native proteins, for instance in the oncoprotein Bcr-Abl.[12] However, the cores of native coiled-coils are constituted predominantly of aliphatic side chains with an almost strict exclusion of aromatic residues. Although there is precedent for incorporation of single aromatic amino acids into coiled-coils with only modest decreases in stability,[13] the effect of totally remodeling the core with bulkier, more rigid side chains has not been explored until recently. Lu and co-workers have described a pentameric 51-residue coiled-coil, named Trp-14, with a core composed entirely of tryptophan.[14] The high-resolution structure showed that most of the indole side chains engage in "knobs-into-holes" packing[15] characteristic of coiled-coils, suggesting that this type of side chain arrangement is tolerant of large sizes and diverse shapes.

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Here we report a novel sequence strategy for programming specific protein−protein interactions, which is based on a coiled-coil motif with a hydrophobic core composed exclusively of phenylalanine residues. The resulting phenylalanine interface self-sorts[16] from a related peptide assembly containing aliphatic residues in the core.

Specificity in coiled-coil binding has been programmed by patterning of polar residues adjacent to the core[17,18] steric matching between core residues,[19] and incorporation of highly fluorinated amino acids to form completely bioorthogonal interfaces.[20] Our current approach is a novel variation which complements and extends the previous examples using only natural amino acids.

Experimental Section

Peptide Synthesis. Peptides were synthesized by both manual *N-tert*-butyloxycarbonyl (*t*-Boc) and automated 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. For *t*-Boc chemistry, standard in situ neutralization protocols were employed, with hydroxybenzatriazole tetramethyluronium hexafluorophosphate (HBTU, Novabiochem, San Diego, CA) as coupling reagent.[21] Fmoc synthesis was performed with an Advanced ChemTech 348 Ω synthesizer (AACEP, Louisville, KY) using diisopropylcarbodiimide and hydroxybenzatriazole as coupling activation reagents. Protected amino acids and peptide synthesis reagents were from Advanced ChemTech (Louisville, KY) and Novabiochem. Cleaved peptides were purified by RP-HPLC using linear gradients of water and acetonitrile (0.1% TFA), and their masses confirmed by MALDI TOF-MS using α-cyano-4-hydroxycinnamic acid (Sigma) or sinapinic acid (Sigma) as matrixes.

Circular Dichroism. Circular dichroism spectra were measured on a JASCO J-715 spectropolarimeter fitted with a JASCO PTC-423S Peltier temperature controller. Concentrations of peptide stock were measured by amino acid analysis or by measuring tryptophan absorbance in 6 M Gdn·HCl (extinction coefficient 5690 $M^{-1}\cdot cm^{-1}$ at 280 nm).[22] Molar ellipticities were calculated using the relation $[\theta] = \theta_{obs}\cdot(MRW)/10\cdot l\cdot c$, where θ_{obs} is the measured signal in millidegrees, MRW is the mean residue molecular weight (molecular weight of the peptide divided by the number of residues), l is the optical path length of the cell in cm, and c is the concentration of peptide in mg/mL. Thermal denaturation curves were obtained at peptide concentrations of 10 μM in 10 mM phosphate at pH 7.4 with 137 mM NaCl and 2.7 mM KCl at 25 °C. Molar ellipticity at 222 nm was measured as a function of temperature in steps of 0.5 °C with an overall temperature change rate of 30−32 °C/h. Data were collected over 8 s per point. The T_m's were determined from the minima of the first derivative of θ_{222} versus T^{-1} where T is the temperature in Kelvin.

Analytical Ultracentrifugation. Apparent molecular masses were determined by sedimentation equilibrium on a Beckman XL-A ultracentrifuge. Peptides were loaded at three different concentrations (see Table S1 in the Supporting Information) in 10 mM phosphate, pH 7.4, 137 mM NaCl, 2.7 mM KCl and equilibrated at rotor speeds of 35 000, 40 000 and 45 000 rpm for 18 h at 10 °C. Absorbance scans were taken at 230 nm and fit to the following equation describing homogeneous single species sedimentation:

$$\mathrm{Abs} = A'\cdot e^{H\cdot M\cdot(x^2-x_0^2)} + E$$

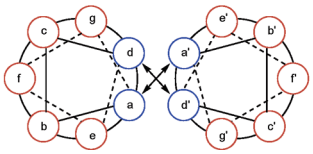
where Abs = absorbance at radius r, A' = absorbance at reference radius x_0, $H = (1 - \bar{V}\rho)\omega^2/2RT$, with $\bar{V}$ = partial specific volume of the peptide, ρ = solvent density = 1.0069, ω = angular velocity in radians/s, M = apparent molecular weight, E = blank absorbance. Data were fit using the nonlinear least-squares method implemented in Igor Pro v4.05 with partial specific volumes and solution densities estimated using SEDNTERP (http://www.rasmb.bbri.org).

Disulfide Exchange. Glutathione exchange was performed as described previously.[20,23] Typical conditions were 10 mM phosphate pH 7.6, 125 μM oxidized glutathione, 450 μM reduced glutathione. Aliquots were removed from the exchange solution and immediately quenched with acid to pH < 2 and injected on analytical RP-HPLC. For exchange using TCEP, disulfide-bonded heterodimer **P1**−**A1** (8 μM in 10 mM MOPS pH 7.8, 137 mM NaCl) was incubated with ∼0.1 equiv of TCEP and allowed to equilibrate over 18−26 h.[24] All experiments were carried out under an inert Ar atmosphere. Aliquots at various times were quenched and analyzed by RP-HPLC. Peaks areas corresponding to each peptide product were determined by manual integration using the Hitachi D-7000 software package. Under the conditions of the experiment, the molar extinction coefficient of $(\mathbf{A1})_2$ was slightly larger than that of $(\mathbf{P1})_2$ ($\epsilon_{\mathbf{A1}}/\epsilon_{\mathbf{P1}} = 1.18$). This was verified and measured by injection of quantitatively reduced **P1**−**A1**. With correction for the difference in molar extinction coefficient, the integration values were used directly to compute K_{redox}. The ΔG_{spec} reported is an average of four experiments. Thermodynamic cycle analysis was carried out as previously described, with the assumption that the peptides are completely folded under the conditions of the experiment.[17]

[A]

	g	a b c d e f g	a b c d e f g	a b c d e f g	a b c d e f g	a b c	
P	Ac-NH-	AQ**F**KKE	**F**QA**F**KKE	**F**AQ**F**KWE	**F**QA**F**KKE	**F**AQ-CONH$_2$	
A	Ac-NH-	AQ**L**KKE	**V**QA**L**KKE	**W**AQ**L**KSE	**V**QA**L**KKE	**V**AQ-CONH$_2$	
P1	Ac-NH-CGG-	AQ**F**KKE	**F**QA**F**KKE	**F**AQ**F**KWE	**F**QA**F**KKE	**F**AQ-CONH$_2$	
A1	Ac-NH-CGG-	AQ**L**KKE	**V**QA**L**KKE	**W**AQ**L**KSE	**V**QA**L**KKE	**V**AQ-CONH$_2$	
P2	Ac-NH-	E **F**KQ**F**KDE	**F**EE**F**KSE	**F**WH**F**KNE	**F**AR**F**KKE	**F**GE-CONH$_2$	

[B]



$(\mathbf{P1})_2$ = **P1**-S-S-**P1**

$(\mathbf{A1})_2$ = **A1**-S-S-**A1**

P1-**A1** = **P1**-S-S-**A1**

Figure 1. (A) Primary sequences of the designed peptides. (B) Helical wheel depiction of a parallel coiled-coil dimer. Oligomer formation is driven by hydrophobic interactions between core *a* and *d* residues.

Results and Discussion

Peptide Design. Figure 1 shows the sequences of the designed phenylalanine construct **P** and the aliphatic peptide **A**. The following design elements were utilized for the construction of a helical bundle with an all aromatic core. In **P**, we incorporated phenylalanine residues in all of the eight core *a* and *d* positions of a previously reported 30-residue prototypical coiled-coil sequence.[20] The lack of a central polar residue was intended to maximize core stability, although in principle it allows the peptide to populate multiple oligomerization states.[25] Charged residues at the *e* and *g* positions were patterned such that the peptide is capable of self-association only in the parallel orientation.[26] A single tryptophan residue allows accurate

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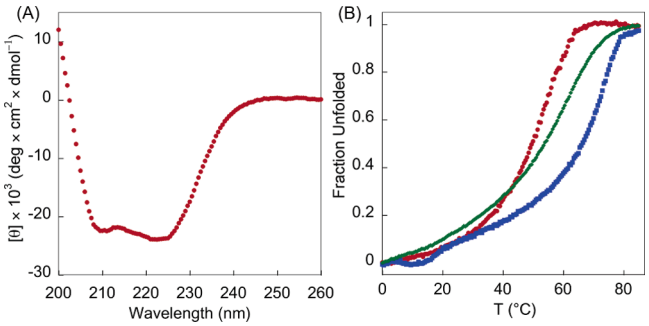

Figure 2. Left panel: circular dichroism spectrum of **P** (14 μM, 15 °C, 10 mM phosphate pH 7.4, 137 mM NaCl, 2.7 mM KCl). Right panel: thermal denaturation of **P** (●), $(\mathbf{A1})_2$ (◆), and $(\mathbf{P1})_2$ (■). For thermal denaturation, [**P**] = 10 μM, $[(\mathbf{P1})_2] = [(\mathbf{A1})_2] = 5$ μM. Buffer conditions are as in the left panel.

measurement of peptide concentration by UV−vis spectroscopy. Peptide **P2** combines the *a*, *d*, *e*, and *g* residues (i.e., the core plus flanking residues) of **P** with the *b*, *c*, and *f* positions from GCN4-p1.[27] It has been previously noted that many coiled-coils incorporating residues from GCN4-p1 at the *b*, *c*, and *f* positions form discrete structures and crystallize readily.[28] Peptide **A1** is derived from the same prototypical sequence as **P1** and is expected to form a parallel coiled-coil with an aliphatic hydrocarbon core. Like **P1**, it has a Cys-Gly-Gly tripeptide appended to the N-terminus. The single Trp substitution at a central *a* position of $(\mathbf{A1})_2$ was included in order to tune and match its thermal stability with that of $(\mathbf{P1})_2$.[13]

Structural Characterization. Sequence comparison between **P** and a database of known coiled-coils through the online MultiCoil interface returned a less than 2% probability of forming a dimeric or trimeric coiled-coil.[29] Nevertheless, **P** exhibits a circular dichroism (CD) spectrum characteristic of α-helical peptides (Figure 2), with a θ_{222} consistent with that of a coiled-coil of this size.[13,30] Thermal denaturation of **P** was cooperative with a T_m of 49 °C (Figure 2) and comparable to peptides with aliphatic cores and a single polar residue.[13a,31] This result is interesting given that mutating small hydrophobic core residues to larger ones tends to destabilize protein folds.[32] Indeed, single leucine-to-phenylalanine mutations have been shown to be destabilizing in model coiled-coils.[13] It seems likely that aliphatic and aromatic residues impose different optimal packing geometries on the coiled-coil backbone, as suggested by the helical interaction parameters observed by Lu *et al.* in Trp-14.[14] We speculate that phenylalanine incorporation at all core positions is more likely to lead to a more stable fold than an intermediate mixture of aliphatic residues with phenylalanine. This also suggests a simplistic evolutionary rationale for the lack of phenylalanine core coiled-coils in nature. Incremental

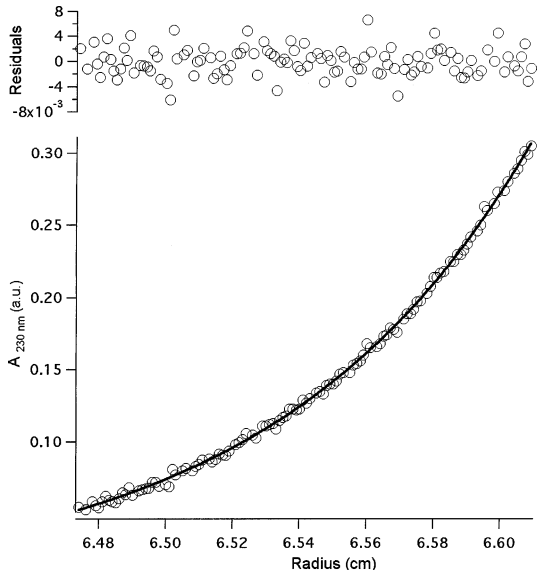

Figure 3. AUC sedimentation equilibrium trace for peptide **P2** (10 μM peptide in 10 mM phosphate, pH 7.4 with 137 mM NaCl and 2.7 mM KCl). The solid line through the data points is a calculated fit for a trimeric aggregate.

mutations in core residues destabilize folds, which is unlikely to confer selective advantages to an organism. Generation of a stable folded mutant would require simultaneous substitution at several adjacent core positions. On the other hand, our results also highlight the robustness of the coiled-coil fold, as it is retained despite a substantial shift in sequence.

As designed, disulfide-bonded peptides $(\mathbf{A1})_2$ and $(\mathbf{P1})_2$ exhibit comparable thermal stabilities as judged by the temperature dependence of the CD signal at 222 nm (Figure 2).

Solution oligomerization behavior of the **P** family of peptides was interrogated by equilibrium analytical ultracentrifugation (AUC-SE). In general, as might be expected for coiled-coils lacking central polar residues, multiple states appear to be populated (Table S1 in the Supporting Information).[31] AUC-SE analysis revealed that **P** sediments with a molecular weight intermediate between dimer and trimer while disulfide-bonded peptide $(\mathbf{P1})_2$ appears to be largely a four-helix bundle. GCN4-p1 analogue **P2** consistently sediments as a trimeric assembly at 10 μM concentration (Figure 3) and may be suitable for structural analysis by X-ray diffraction. We note that while the data presented here do not strictly exclude other multimeric, α-helical structures, a coiled-coil structure is strongly suggested by the cumulative evidence.

AUC-SE of the disulfide-bonded $(\mathbf{A1})_2$ shows that the peptide is largely dimeric but higher oligomerization states are also populated (see the Supporting Information).

Oligomerization Specificity. To further characterize the oligomerization interface presented by **P**, we performed a disulfide exchange assay between the peptide variants **P1** and **A1**.[23] Disulfide-bound **P1**−**A1** heterodimer can be prepared by air oxidation of a mixture of free thiol peptides in 6 M guanidinium hydrochloride solution. Initial disulfide exchange experiments with standard glutathione exchange buffers showed, upon quenching and HPLC analysis, disproportionation of heterodimeric **P1**−**A1** into disulfide-bound homodimers $[(\mathbf{P1})_2$ and $(\mathbf{A1})_2]$. Identical product mixtures were observed upon mixing the two free thiol peptides under similar buffer conditions, indicating that equilibrium was reached. However, in both

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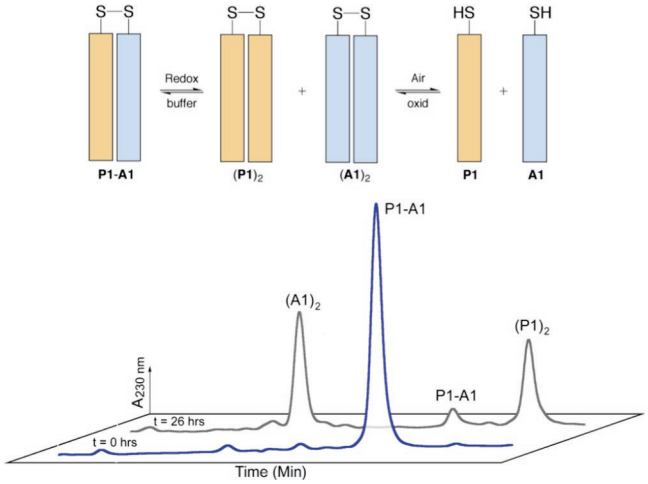

Figure 4. Preferential homodimer formation by aromatic and hydrocarbon cores. HPLC chromatograms at 0 and 26 h after incubation of heterodimer with TCEP (0.1 equiv). The peak areas were integrated manually to afford the relative amounts of each species for a thermodynamic cycle analysis.

cases up to ~35% of both peptides eluted as glutathione dimers, and we were unable to curtail this trend by varying the peptide or glutathione concentration or the reduced/oxidized ratio of the glutathione buffer.[33] Kinetic analysis of coiled-coil association suggests that strand exchange occurs on the time scale of seconds to minutes.[34] We hypothesized that a small amount of free thiol peptide mixed with heterodimer would catalyze the formation of an equilibrium mixture of disulfide-bound peptides. The exchange experiment was therefore repeated by introducing <0.1 equiv of the reductant tris(carboxyethyl-)phosphine hydrocholoride (TCEP) to the heterodimer. (Figure 4). Again, homodimers were preferred, with <3% of the starting **P1**−**A1** heterodimer remaining. From the relative peak areas at equilibrium, the free energy of specificity for the formation of homodimers, ΔG_{spec}, is calculated to be -1.5 ± 0.1 kcal/mol,[35] comparable with that of previously reported self-sorting coiled-coils.[17,20]

Conclusions

We have demonstrated that the core residues of a coiled-coil can be radically retooled resulting in peptides with orthogonal binding specificity but the same overall fold. Earlier studies from our laboratory and others have shown that fluorocarbon amino acids can direct hyperstable and highly specific interactions when incorporated into the cores of soluble coiled-coil and membrane-embedded peptides.[20,36,37] Here we report comparable binding specificity from a core of entirely natural amino acids. We anticipate that this motif may be useful for the design of novel signaling proteins, especially since native coiled-coils appear not to make use of this motif. We are therefore working on the optimization of this sequence to provide a specific oligomerization state. We are also interested in whether this motif might direct the oligomerization of transmembrane α-helices and are conducting searches of genomic databases for biological precedent.

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Supporting Information Available: Experimental procedures, accompanying analytical data, and complete author list for ref 1a (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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