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Strong Contributions from Vertical Triads to Helix-Partner Preferences in Parallel Coiled Coils

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Supporting Information

ABSTRACT: Pairing preferences in heterodimeric coiled coils are determined by complementarities among side chains that pack against one another at the helix−helix interface. However, relationships between dimer stability and interfacial residue identity are not fully understood. In the context of the "knobs-into-holes" (KIH) packing pattern, one can identify two classes of interactions between side chains from different helices: "lateral", in which a line connecting the adjacent side chains is perpendicular to the helix axes, and "vertical", in which the connecting line is parallel to the helix axes. We have previously analyzed vertical interactions in antiparallel coiled coils and found that one type of triad constellation ($a'-a-a'$) exerts a strong effect on pairing preferences, while the other type of triad ($d'-d-d'$) has relatively little impact on pairing tendencies. Here, we ask whether vertical interactions ($d'-a-d'$) influence pairing in *parallel* coiled-coil dimers. Our results indicate that vertical interactions can exert a substantial impact on pairing specificity, and that the influence of the $d'-a-d'$ triad depends on the lateral a' contact within the local KIH motif. Structure-informed bioinformatic analyses of protein sequences reveal trends consistent with the thermodynamic data derived from our experimental model system in suggesting that heterotriads involving Leu and Ile are preferred over homotriads involving Leu and Ile.

Coiled-coil interactions are among the most common motifs in protein tertiary and quaternary structure. Sequences that participate in coiled coils usually display a heptad repeat of the form HPPHPPP, in which residues at H positions are hydrophobic, and residues at P positions are polar. Heptad positions are typically designated *abcdefg*;[1] upon α-helix formation, the H positions (a and d) become aligned in a stripe roughly parallel to the helix axis. Coiled-coil association is driven by burial of hydrophobic stripes against one another, with a characteristic "knobs-into-holes" (KIH) interdigitation of the H side chains from partner helices at the interface.[2] Considerable effort has been devoted to identifying the "rules" that govern coiled-coil partner preferences, because this motif represents a relatively straightforward manifestation of the general "protein-folding" problem (i.e., prediction of structure from sequence),[3] and because of interest in developing orthogonal pairs of coiled-coil-forming sequences for engineering applications.[4] Although some key factors have been delineated, it remains difficult to predict or rationalize stability differences among alternative coiled-coil pairs.

The work described here focuses on a previously unexplored partner-specifying factor in parallel coiled-coil formation. Figure 1 illustrates the KIH side-chain interdigitation in a generic parallel coiled-coil dimer interface. One a side chain (green) is laterally paired with an a' side chain (blue) from the partner helix. Extensive studies by Vinson et al. and Hodges et al. have established that the identity of a influences the preference for a'.[5]

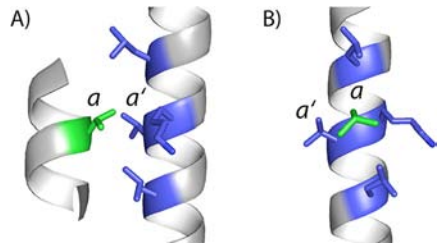

Figure 1. Orthogonal views of an a knob (green)-into-hole (blue residues) interaction from an experimentally determined protein structure (PDB 2ZTA). Images were generated using PyMOL.

Limited work indicates that $d-d'$ lateral pairing provides a further source of partner preferences.[6] In addition, Coulombic complementarity is important between acidic and basic residues that are often found to flank the hydrophobic $a-d$ core, i.e., at $e-g'$ and $e'-g$ pairs that become juxtaposed upon dimer formation.[7] These pairings may be described as "vertical", since a line connecting the two side chains runs approximately parallel to the helix axes. Here, we evaluate the importance of vertical interactions among hydrophobic core residues at a and d sites, an issue that has not previously been addressed for parallel coiled coils.

Recently, we examined vertical interactions among hydrophobic core positions in *antiparallel* coiled-coil dimers, via both experimental analysis of model systems and bioinformatic analysis of the protein structure database.[8] In this case there are two possible vertical triad arrangements, $a'-a-a'$ and $d'-d-d'$. Our results indicated that a triads exert a substantial effect on antiparallel coiled-coil partner preference,[8a] but that d triads have a relatively small impact on pairing.[8b] In light of this position-dependent distinction, it became important to examine vertical

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triads in parallel coiled coils. The results reported below reveal a substantial effect of $d'-a-d'$ vertical triads on parallel coiled-coil pairing; in addition, we find an interplay between the identity of the a' lateral partner of the central a residue and the stability associated with a given vertical triad.

Our study began with the design of a dimeric, parallel coiled-coil model system that was amenable to variation at d positions (i.e., $d \neq$ Leu). Previously we developed a heterodimeric parallel coiled-coil model system that allowed exploration of sequence-stability relationships at $a-a'$ lateral contact sites.[9] Despite many design iterations, however, mutation of d positions in this background always led to aggregation in pH-neutral aqueous buffer. Therefore, we turned to the alternative design shown in Figure 2, in which positions a, a', d'_1, and d'_2 are sites of variation,

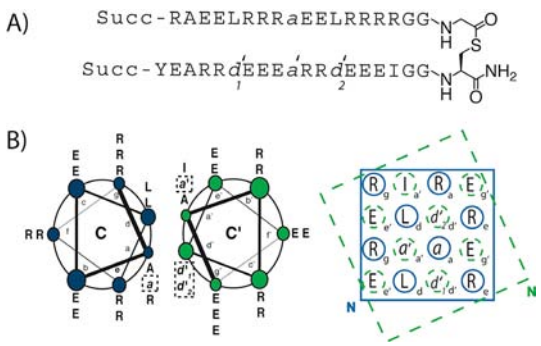

Figure 2. (A) Sequence of parallel coiled-coil model system $\mathbf{C_T}$-$\mathbf{C'_{SG}}$ (SG = second generation). Succ = *N*-terminal succinyl group. (B) Helical wheel and net diagrams of $\mathbf{C_T}$-$\mathbf{C'_{SG}}$.

with the remaining a and d positions occupied by Ala, Ile, or Arg. We placed Arg at a C-terminal a position with the goal of promoting two-helix stoichiometry by discouraging higher-order interactions.[10] All b, c, e, f, and g positions are occupied by either Arg or Glu, and these residues are arranged to maximize possible intra- and interhelical ion pairs upon coiled-coil formation.[11]

Parallel helix orientation is promoted by a thioester bond between the C-terminus of one segment and the side chain of a C-terminal Cys residue on the other. This design allows us to monitor coiled-coil stability under native conditions using thiol–thioester exchange.[12] Initial physical characterization of our heterodimeric coiled-coil design was carried out for $a = a'$ = Ile and $d'_1 = d'_2$ = Leu using a disulfide-linked analogue ($\mathbf{C_{S\text{-}S}C'_1}$), because the thioester itself ($\mathbf{C_T}$-$\mathbf{C'_{SG}}$) suffers hydrolysis over the time period required to perform sedimentation equilibrium studies. Disulfide-linked analogue $\mathbf{C_{S\text{-}S}C'_1}$ was prepared by changing the final Gly residue of the basic segment to Cys, and then forming the heterodisulfide. Sedimentation equilibrium analytical ultracentrifugation (SEAUC) data gathered at three concentrations (50, 100, and 150 μM) indicate that $\mathbf{C_{S\text{-}S}C'_1}$ does not self-associate under conditions similar to those used for thioester exchange assays.[13] The far UV circular dichroism (CD) spectrum for $\mathbf{C_{S\text{-}S}C'_1}$ is consistent with extensive α-helicity, with minima at 208 and 222 nm. Physical examination of four additional disulfide-linked peptides ($\mathbf{C_{S\text{-}S}C'_{2-5}}$) containing mutations at both a and d positions gave analogous CD and SEAUC results.[13] These control experiments are vital because they allow us to interpret the thioester exchange equilibrium constant (K_{CC}) determined for different versions of $\mathbf{C_T}$-$\mathbf{C'_{SG}}$ strictly in terms of intramolecular coiled-coil formation.

Because the behavior of $\mathbf{C_{S\text{-}S}C'_{1-5}}$ suggested that this parallel coiled-coil design tolerates substitutions at a and d positions, we undertook thioester exchange studies with the $\mathbf{C_T}$-$\mathbf{C'_{SG}}$ system to examine interplay between mutations in each helical segment. We assume that upon coiled-coil formation a traditional KIH packing interface is generated within $\mathbf{C_T}$-$\mathbf{C'_{SG}}$. Residue a acts as a knob that packs into vacant space generated by residues a', d'_1, d'_2, and g' of the partner helix (Figures 1 and 2; g' is Glu9 of the lower peptide strand in Figure 2A). We evaluated all homo- and heterotypic $a-a'$ contacts that result from placement of Ile, Leu, Val, Ala, or Asn at these two positions (25 combinations) while holding d'_1 and d'_2, the vertical partners of a, constant as Leu.[13] Trends observed in this data set are consistent with the findings of Vinson et al. for $a-a'$ pairings in a different parallel coiled coil.[5,13] These results indicate that our $\mathbf{C_T}$-$\mathbf{C'_{SG}}$ design provides quantitative insight regarding sequence-stability relationships in the parallel coiled coil dimer motif.

A second round of thioester exchange studies was carried out involving five peptide thiols in which a' was varied among Ile, Leu, Val, Ala and Asn, with $d'_1 = d'_2$ = Ile, paired with the five peptide thioesters used for the first studies (a = Ile, Leu, Val, Ala or Asn).[13] Comparing these data to those obtained with peptide thiols in which $d'_1 = d'_2$ = Leu allows us to assess whether the difference between Leu and Ile at the d' positions in the $d'-a-d'$ vertical triad motif has a significant effect on parallel coiled-coil stability. Changes are evident from comparisons of appropriate data points, but direct comparison of the two data sets is complicated by the fact that altering d'_1 and d'_2 leads to multiple pairing changes at the hydrophobic interface.

To isolate the energetic significance of changes in the $d'-a-d'$ vertical triads for d' = Leu vs Ile, we consider ΔG_{CC} values for a set of four mutants of $\mathbf{C_T}$-$\mathbf{C'_{SG}}$, involving two different residues at position a and a constant residue at a'.[8] This approach is illustrated in Figure 3 for a = Ile or Leu and a' = Ile. The pair of

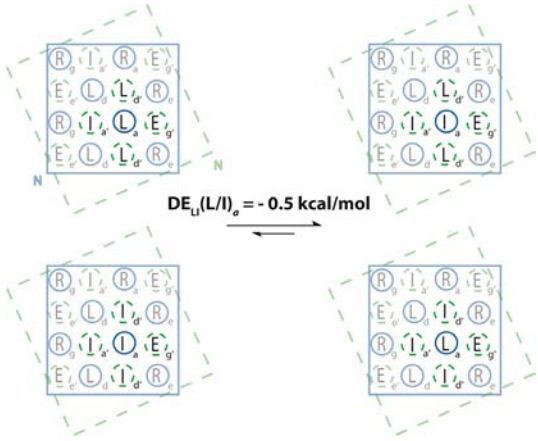

Figure 3. Partial helical net diagrams for $\mathbf{C_T}$-$\mathbf{C'_{SG}}$ and three mutants used to calculate the discrimination energy (DE). The DE value was derived from the thermodynamic data in Table S2 (Supporting Information).

coiled coils on the left has a = Leu with vertical partners d' = Leu, and a = Ile with vertical partners d' = Ile (i.e., Leu–Leu–Leu vertical triad and Ile–Ile–Ile vertical triad). The pair of coiled coils on the right has a = Ile with vertical partners d' = Leu, and a = Leu with vertical partners d' = Ile (i.e., Leu–Ile–Leu vertical triad and Ile–Leu–Ile vertical triad). Careful examination of the helical-net diagrams in Figure 3 shows that the position of this hypothetical equilibrium should depend solely on the energetic difference between the two sets of vertical triads centered on the

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a positions. The resulting ΔG value, which we term the "discrimination energy" (DE), indicates the extent to which one set of vertical triads is favored over the other. We designate this energy term $DE_{LI}(L/I)_a$ to indicate that (1) we are considering Leu and Ile as the alternative vertical residue partners (LI subscript); (2) on the left Leu is placed between Leu vertical partners while Ile is placed between Ile vertical partners (L/I in parentheses); and (3) the knob residue in the vertical triad is an *a* position in the heptad sequence repeat. ΔG_{CC} values for the four coiled-coil variants involved in the hypothetical equilibrium in Figure 3 indicate that $DE_{LI}(L/I)_a = -0.5$ kcal/mol when a' = Ile. Thus, in this case, the vertical heterotriads are favored over the vertical homotriads by 0.5 kcal/mol. Similar values of $DE_{LI}(L/I)_a$ are observed for other hydrophobic residues at a', as indicated below, which suggests that the preference for Leu/Ile heterotriads over homotriads should be a general feature of dimeric parallel coiled coils.

Previously we have compared trends observed in thioester exchange assays to bioinformatic analysis of natural coiled-coil structures using the CC+ database (http://coiledcoils.chm.bris.ac.uk/ccplus/search/).[14] This Web-based resource was created to allow exploration of sequence-structure relationships among coiled coils. As we contemplated using CC+ to assess the relative favorability of $d'-a-d'$ vertical homo- and heterotriads composed of Leu and/or Ile, we were concerned that the strong prevalence of Leu at *d* positions in parallel coiled-coil dimers would exclude meaningful statistical analysis. Indeed, Leu occupies 528 of 1172 *d* positions in parallel coiled-coil dimers in a subset of the database.[13,15] Therefore, we turned our attention to *sequence* databases to broaden our search space. We used the full-length sequences of each non-identical, parallel, heterodimeric, same-chain (i.e., both helical segments are in the same polypeptide chain) coiled-coil sequence as a seed for a sensitive, iterative profile Hidden Markov Model (HMM) comparison search of the Uniprot[16] protein sequence database using HHblits.[17] This seed was chosen to mimic our designed coiled-coil-model system and the folding event we detect (i.e., intramolecular coiled-coil formation) in thioester exchange assays. The sequences returned were culled so that no two shared more than 50% sequence similarity using CD-HIT,[18] and then realigned using Muscle with standard settings.[13,19] Sequences that aligned to the coiled-coil regions of the seed sequence were extracted, and coiled-coil-register positions were assigned from the seed. From the alignment, residue distributions at the relevant register positions were examined and KIH interactions inferred.[20] Table 1 gives the observed and expected counts for each of the residues at the central *a* position, with both alternative vertical backgrounds, $d' = d'$ = Leu or $d' = d'$ = Ile.

Expected counts for each combination were calculated on the basis of the frequencies of these residues (i.e., Leu or Ile) at *a* positions relative to the total number of inferred KIH interactions uncovered using HHblits. Defining all three residues in the $d'-a-d'$ vertical triad limited the counts returned from the search; nevertheless, the data were sufficient to perform reliable analyses and to draw conclusions. The ratio of observed/expected (O/E) is effectively a propensity for a side chain to be in a specified vertical contact environment. When the vertical background is Leu ($d' = d'$ = Leu) we observed a preference for Ile at *a* (O/E = 1.3), suggesting the Leu−Ile−Leu heterotriad is preferred over the Leu−Leu−Leu homotriad. When the vertical background is Ile ($d' = d'$ = Ile) both O/E values were greater than 1; however, the value for the Leu at *a* was significantly

Table 1. Bioinformatic Analysis[a]

A)

	$d' = d'$ = *Leu*		
Amino acid at *a*	Observed	Expected	Observed/Expected
Ile	67	50.8	1.3
Leu	119	126	0.9

B)

	$d' = d'$ = *Ile*		
	Observed	Expected	Observed/Expected
Ile	13	11.1	1.2
Leu	41	27.5	1.5

[a]Numbers of specific $d'-a-d'$ combinations uncovered through use of a set of full-length sequences of each non-identical, parallel, heterodimeric, same-chain coiled-coil sequence as a seed for an iterative profile Hidden Markov Model (HMM) comparison search of the Uniprot protein sequence database using HHblits.

greater than 1 (O/E = 1.5), again indicating a preference for heterotriads. Taken together, these data suggest that while homotriads are not disfavored (O/E is not significantly less than 1.0), the heterotriads are preferred, a conclusion consistent with results obtained using our experimental model system.

The correlation we observed for Leu-Ile-Leu and Ile-Leu-Ile vertical heterotriads provided impetus for further analysis of the thioester exchange data.[13] By definition $DE_{LI}(X/Y)_a = -DE_{LI}(X/Y)_a$, and because $DE_{LI}(X/Y)_a$ is meaningless if X = Y, 10 independent $DE_{LI}(X/Y)_a$ values can be calculated when X and Y each vary among the five amino acids residues Ile, Leu, Val, Ala, and Asn. The number of accessible $DE_{LI}(X/Y)_a$ values expands by a factor of 5 because we have examined five partner lateral contacts (i.e., a' = Ile, Leu, Val, Ala, or Asn). Taken together, the thioester exchange data allow for the determination of 50 unique $DE_{LI}(X/Y)_a$ values, and allow us to probe whether the significance of a vertical $d'-a-d'$ triad depends on the lateral partner (a') of the *a* residue that resides within that triad (Table 2). We consider discrimination energy to be significant if the absolute DE_{LI} value $\geq$0.4 kcal/mol, which is twice the estimated experimental uncertainty. Using this standard 33 of 50 $DE_{LI}(X/Y)_a$ values (66%) are significant, a result that indicates amino acids other than Leu and Ile can exert an impact on dimerization selectivity.

Examination of the data set leads to a few notable observations. First, the identity of the lateral partner of the *a* residue in the $d'-a-d'$ vertical triad can determine whether the discrimination energy is significant. (A similar trend was observed in analogous studies of an antiparallel coiled-coil dimer.[8]) For example, $DE_{LI}(V/L)_a = 0.6$ when the lateral a' contact is Ile, but $DE_{LI}(V/L)_a = 0.2$ when the lateral a' contact is Leu. In some cases, however, $DE_{LI}(X/Y)_a$ is significant across a variety of lateral a' contacts. For example, $DE_{LI}(L/I)_a \geq 0.4$ when the lateral a' contact is any amino acid bearing a hydrocarbon side chain (i.e., Ile, Leu, Val, Ala); and only when a' = Asn is the discrimination energy insignificant. The lack of a simple rule to describe pairing specificity trends in our data set highlights the need for fundamental sequence-stability studies of the type reported here.

We have described a new parallel dimeric coiled-coil-model system to study sequence variation at *d* positions in the heptad repeat without problems from higher-order association. We have used this model system to explore the significance of $d'-a-d'$ vertical triads at the helix−helix interface. Our thioester exchange experiments indicate a significant preference for Leu−Ile−Leu and Ile−Leu−Ile vertical heterotriads over Leu−Leu−Leu and Ile−Ile−Ile vertical homotriads. A comparable preference for vertical heterotriads is observed in the Uniprot protein sequence

Table 2. Discrimination Energy ($DE_{LI}(X/Y)_a$) Values (kcal/mol)[a]

a' = Ile

Y \ X	Ile	Leu	Val	Ala	Asn
Ile		-0.5	0.1	-0.2	-1.0
Leu			0.6	0.3	-0.5
Val				-0.3	-1.1
Ala					-0.8
Asn					

a' = Leu

Y \ X	Ile	Leu	Val	Ala	Asn
Ile		-0.5	-0.3	-1.0	-1.2
Leu			0.2	-0.5	-0.7
Val				-0.7	-0.9
Ala					-0.2
Asn					

a' = Val

Y \ X	Ile	Leu	Val	Ala	Asn
Ile		-0.6	-0.2	-0.8	-1.4
Leu			0.4	-0.2	-0.8
Val				-0.6	-1.2
Ala					-0.6
Asn					

a' = Ala

Y \ X	Ile	Leu	Val	Ala	Asn
Ile		-0.6	-0.1	-1.3	-0.8
Leu			0.5	-0.7	-0.2
Val				-1.2	-0.7
Ala					0.5
Asn					

a' = Asn

Y \ X	Ile	Leu	Val	Ala	Asn
Ile		0.0	0.0	0.0	0.7
Leu			0.0	0.0	0.7
Val				0.0	0.7
Ala					0.7
Asn					

[a]The $DE_{LI}(X/Y)_a$ values were obtained from thioester exchange data for C_T-C'_{SG} mutants. Symbols X and Y represent the position labeled *a* in the upper peptide strand in Figure 2A. See the Supporting Information for a general representation of Figure 3 that describes how to calculate $DE_{LI}(X/Y)_a$.

database when using same-chain, parallel, heterodimeric coiled coils as seed sequences, which implies that the preferences reported by our model system reflect preferences manifested among evolved proteins. Our analysis also reveals a significant energetic interplay between the vertical and lateral side chains that make up a "knobs-into-holes" motif.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, peptide characterization, CD and AUC data, HPLC chromatograms of thioester exchange assays, and bioinformatics data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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