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Side-Chain Pairing Preferences in the Parallel Coiled-Coil Dimer Motif: Insight on Ion Pairing between Core and Flanking Sites

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Proteins collectively display a broad array of tertiary and quaternary structures, with many different modes of packing between neighboring secondary structure elements. Among the possibilities, the α-helical coiled coil is unusual in that it is both common and regular.[1] In the simplest case, two α-helices associate side-by-side, wrapping around one another with a slight left-handed superhelical twist. A characteristic "knobs-into-holes" interdigitation of side chains is observed at the helix−helix interface, whether the helices are parallel or antiparallel.[2] The relative simplicity of this architecture has led to extensive exploration of sequence−stability relationships,[3] motivated by the prospects of predicting coiled-coil structure from sequence information alone, refining computational tools, and using coiled coils as building blocks in rational protein design and synthetic biology.[4] Although some principles that govern coiled-coil stability have been elucidated, our understanding remains incomplete.

Here we introduce a heterodimeric parallel coiled-coil model system designed to provide new insights on the origins of stability and helix-pairing preferences. Our system employs relatively short peptide segments (20 or 21 residues), which facilitates broad exploration of sequence variations. Parallel, two-helix assembly is promoted by a thioester linkage between the C-terminus of one segment and the side chain of a C-terminal Cys residue on the other; this design enables us to monitor coiled-coil stability under native conditions via thiol−thioester exchange equilibration.[5] Mutation and thermodynamic analysis generate hydrophobic side-chain pairing preferences at the helix−helix interface that agree well with results previously obtained by Vinson et al. with larger heterodimers comprising 96-mer proteins.[3a] Furthermore, an analysis of ionizable side chains at the interface offers new insight on contributions of Coulombic interactions to coiled-coil stability and pairing preferences.

Our experimental design (Figure 1) is based on well-known characteristics of sequences that form coiled coils. The segments intended to adopt α-helical conformations feature a heptad sequence repeat pattern (*abcdefg*), in which side chains at *a* and *d* dominate the helix−helix contacts. Two-helix stoichiometry (rather than alternate three- or four-helix assemblies) is directed by placing Leu at the *d* sites, Ile at the N-terminal *a* positions, and Asn at the *a* sites closest to the covalent connection.[6,7] The remaining (central) *a* positions of each segment (designated X and Ψ) are "guest" sites for substitutions that allow us to probe the impact of paired mutations on coiled-coil stability. The positions that flank the helix−helix interface, *e* and *g*, are occupied exclusively by Arg in one segment and by Glu in the other. Upon intramolecular coiled-coil formation (Figure 1B), these side chains can form interhelical ion pairs (*e*−*g*′ and *e*′−*g*; the primes indicate sites on different helices). The remaining positions (*b*, *c*, and *f*) are occupied by nonionizable residues with high helical propensity[8] but low hydrophobicity.

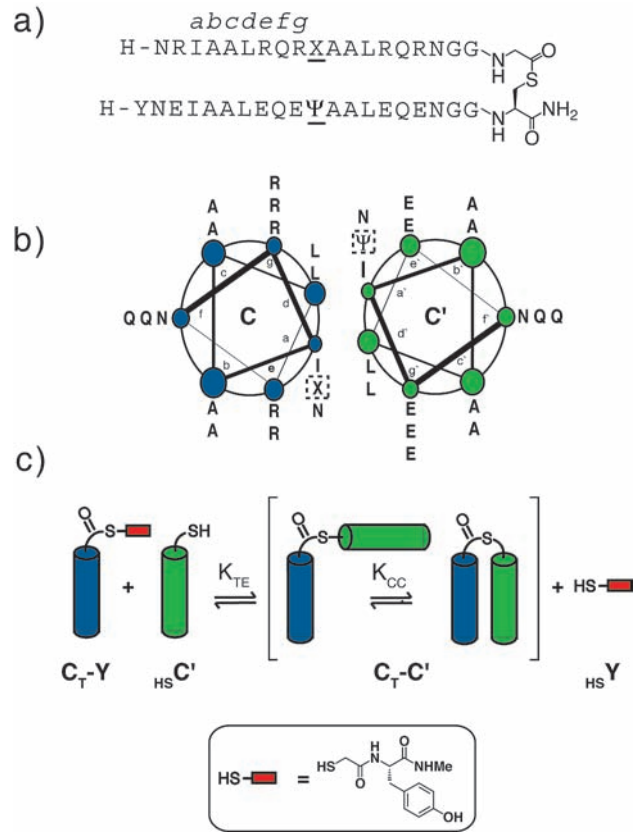

Figure 1. (a) Primary sequence of $\mathbf{C_T}-\mathbf{C}'$ (X = Ψ = Ile). (b) Helical wheel diagram showing the proposed helical regions of $\mathbf{C_T}-\mathbf{C}'$. Guest sites are highlighted in dashed boxes. (c) Cartoon illustrating the thiol−thioester exchange process.

Our heterodimeric coiled-coil design was characterized physically for X = Ψ = Ile using a disulfide-linked analogue ($\mathbf{C_{S-S}C}'$), because the thioester version ($\mathbf{C_T}-\mathbf{C}'$) suffered hydrolysis over the time period required for measurements (e.g., multiple days for sedimentation equilibrium analytical ultracentrifugation (AUC)). $\mathbf{C_{S-S}C}'$ was generated by replacing the final Gly residue of the basic segment with Cys and then forming the heterodisulfide. Sedimentation equilibrium AUC and circular dichroism (CD) spectra recorded for different concentrations for $\mathbf{C_{S-S}C}'$ show that this molecule does not self-associate under conditions used for thioester exchange.[9] The far-UV CD spectrum for $\mathbf{C_{S-S}C}'$ is consistent with extensive α-helicity, which is disrupted upon heating.[9] CD spectra for peptides corresponding to either the basic or acidic segment in $\mathbf{C_{S-S}C}'$ indicate that these fragments are largely unfolded in isolation.[9]

Thiol−thioester exchange (TE) was initiated for X = Ψ = Ile by mixing $\mathbf{C_T}-\mathbf{C}'$ and $_{\mathbf{HS}}\mathbf{Y}$, or by mixing $\mathbf{C_T}-\mathbf{Y}$ and $_{\mathbf{HS}}\mathbf{C}'$ (Figure

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1C), in aqueous buffer (pH 7). Equilibrium is achieved in ~2 h, with $K_{TE} = 158$. This value does not change when the starting component concentrations are varied between 50 and 300 μM. We have shown previously that the folding equilibrium constant for intramolecular coiled-coil formation (K_{CC}) should be equal to $K_{TE} - 1$ if there is no significant packing interaction between the Tyr residue of the $_{HS}$**Y**-derived fragment and the remainder of the molecule in $\mathbf{C_T}-\mathbf{Y}$ and if the thioester bonds in $\mathbf{C_T}-\mathbf{Y}$ and $\mathbf{C_T}-\mathbf{C}'$ are isoenergetic. Control experiments suggest that these conditions are approached in the present system.[9] $K_{TE} = 158$ translates to a favorable free energy (ΔG_{CC}) of −3.0 kcal/mol for intramolecular coiled-coil formation in $\mathbf{C_T}-\mathbf{C}'$, relative to an unfolded state that lacks tertiary contacts.

a)

ΔG_{CC} values obtained by thioester exchange

	Ψ = Ile	Ψ = Leu	Ψ = Val	Ψ = Ala	Ψ = Asn
X = Ile	-3.0	-2.8	-2.5	-2.0	-0.9
X = Leu	-2.9	-2.3	-2.5	-1.2	-0.8
X = Val	-2.7	-2.4	-2.1	-1.4	-0.6
X = Ala	-2.0	-1.2	-1.4	-0.5	-0.8
X = Asn	-0.5	-0.7	-0.5	-0.5	-0.8

Values in kcal/mol
Uncertainty ~ ±0.1 kcal/mol

b)

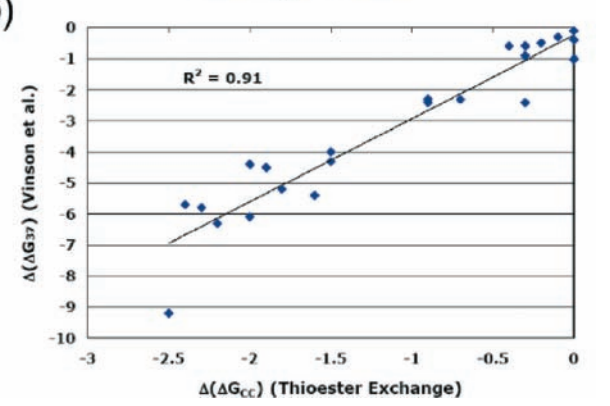

Figure 2. (a) Thermodynamic data (ΔG_{CC}) values determined from thioester exchange of $\mathbf{C_T}-\mathbf{C}'$ mutants. Assays were conducted in 50 mM sodium phosphate buffer (pH 7) with 2 mM TCEP at 25 °C. (b) Correlation diagram comparing $\Delta(\Delta G)$ data from the Vinson laboratory[3a] and our work using thioester exchange. $\Delta(\Delta G)$ values are normalized to the Ala-Ala homotypic pairing.

Upon intramolecular coiled-coil formation by $\mathbf{C_T}-\mathbf{C}'$, the residues at guest sites X and Ψ should be laterally paired at the interhelical interface. We used our system to evaluate the energetics of all 25 $a-a'$ pairings involving Ile, Leu, Val, Ala, and Asn (Figure 2a). Replicate analysis (at least duplicate) was conducted for each mutant. Standard deviations were calculated to arrive at the reported uncertainty of ±0.1 kcal/mol, though the precision of the measurement is higher (±0.03 kcal/mol) in many cases. In general, the uncertainty associated with ΔG_{CC} becomes larger when K_{TE} approaches unity, because K_{CC} approaches zero ($K_{CC} \sim K_{TE} - 1$).

Vinson et al. have reported an extensive evaluation of $a-a'$ pairing energetics, based on thermal denaturation of intermolecular heterocoiled coils formed between designed 96-residue proteins.[3a] Despite deriving from significantly different model system designs (primary sequence, length, inter- vs intramolecular association) and methods for extracting thermodynamic parameters (thermal denaturation vs thioester exchange), the two data sets appear to correlate well (Figure 2b). This correlation indicates that our model system provides quantitative insight regarding sequence−stability relationships for parallel coiled-coil structure.

We applied the TE approach to examine a factor that has recently been suggested to influence coiled-coil stability and selectivity.[10] Based on extensive evaluation and modeling of natural and designed coiled-coil pairs, Keating et al. drew the unexpected conclusion that $a-g'$ and $a'-g$ ion pairs may exert a substantial effect on coiled-coil specificity. This suggestion is noteworthy because interactions involving ion pairing at flanking sites ($e-g'$ and $e'-g$) are widely understood to confer pairing specificity, but the occurrence of ionic side chains at core positions (a or d) has generally been regarded as simply destabilizing, although such residues can be important in specifying the oligomer state.[11]

We explored the energetic significance of $a-g'$ ion pairing by examining X = Glu vs Arg for Ψ = Ile. This mutational analysis explores two unique cases. In the first case the intrahelical $a-e$ ion pair is attractive, while the interhelical $a-g'$ ion pair is repulsive (Figure 3a). This side-chain arrangement should increase α-helicity in the isolated helix-prone segment[12] but *discourage* coiled-coil formation due to the repulsive $a-g'$ ion pair. In the second case the situation is reversed: the intrahelical $a-e$ ion pair is repulsive, while the interhelical $a-g'$ ion pair is attractive (Figure 3b). This side-chain arrangement should *encourage* coiled-coil formation by allowing the a side chain to ion pair with the g' side chain. Comparable possibilities occur for Ψ = Glu vs Arg for X = Ile, as indicated in Figure 3c−d.

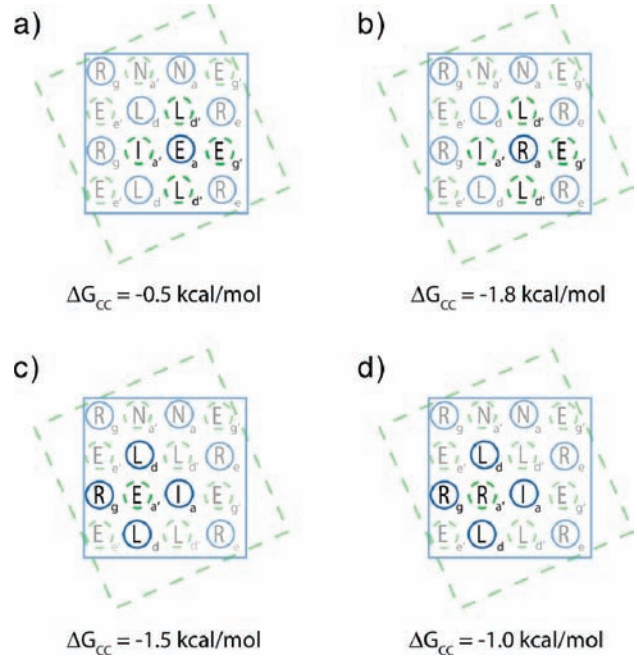

Figure 3. Partial helical net diagrams and ΔG_{CC} values for different $a-g'$ or $a'-g$ ion pairs.

Compared to X = Ψ = Ile ($\Delta G_{CC} = -3.0$ kcal/mol), all four variants indicated in Figure 3 are destabilized, as expected for placement of an acidic or basic side chain at the helix−helix interface.[3a] However, the data clearly show that stability is greater when the interfacial side chain has the possibility to form an $a-g'$ or $a'-g$ ion pair (X = Arg or Ψ = Glu) than when the $a-g'$ or $a'-g$ pairing leads to Coulombic repulsion (X = Glu or Ψ = Arg; compare Figure 3a with 3c, and 3b with 3d). The ΔG_{CC} values for the mutants shown in Figure 3 are independent of concentration when the starting components are varied between 50 and 300 μM. Sedimentation equilibrium AUC of the heterodisulfide correspond-

ing to Figure 3b indicates that this molecule does not self-associate under conditions used for thioester exchange.[9] This observation is consistent with other AUC studies on coiled coils containing a charged residue at an *a* position.[11,13] These control experiments indicate that intermolecular interactions (i.e., self-association of the coiled coil) do not influence the ΔG_{CC} measured for this set of mutants. Analogous stability trends were obtained for the examples in which the nonpolar residue at either X or Ψ is Leu, rather than Ile as shown in Figure 3.[9] Overall, these data indicate that interhelical Coulombic interactions between *a* and *g′* influence coiled-coil stability. Our findings support and amplify recent conclusions that *a*−*g′* charge complementarity can exert a substantial influence on coiled-coil pairing specificity.[10]

To test this hypothesis further, we used the CC+ database[14] to identify a set of parallel dimeric coiled coils in the protein data bank (PDB) that had either Arg or Glu at *a*. While the numbers of examples are small, 24 and 19 respectively, inspection of the structures is informative. A number of *a*−*g′* interactions are observed, but the data set reveals more generally that Coulombic interactions of side chains can involve a variety of two- and three-component combinations. In the case of Arg-Glu ion pairs in which one partner resides at an *a* site, interhelical *a*−*g′* and intrahelical *a*−*e* Coulombic interactions are detected, along with more complex salt-bridged networks.[9] Comparing the $C^{\zeta}-C^{\delta}$ distances for these Arg-Glu pairs gives averages of 4.76 Å for *a*−*g′* pairs ($n = 14$, SD = 0.69 Å) and 5.19 Å for *a*−*e* pairs ($n = 13$, SD = 0.77 Å). The mean values of the distances might suggest that *a*−*g′* pairs lead to "better" interactions than the *a*−*e* alternatives, but for this limited data set we can confidently conclude only that interhelical *a*−*g′* salt bridges are at least as good as intrahelical salt bridges between *a* and *e*.

The contribution of *a*−*g′* Coulombic interactions to coiled-coil stability and selectivity noted by Keating et al.[10] and supported by our observations is not reflected in data acquired for the 96-mer heterodimers.[3a] In the Vinson system, Arg is always energetically superior, or at least comparable, to Glu at a guest *a* site, whether the spatially adjacent *g′* site is occupied by Arg or Glu, so long as the lateral (*a′*) partner is nonpolar. However, there is a significant difference between the two systems: our design contains no ionizable side chains further out from the coiled-coil interface, particularly at *b* or *c* positions, but the Vinson system has several ionizable side chains in these positions. Therefore, it is possible that alternative side-chain ion-pairing patterns, or indeed higher-order networks of the types detected in our structural survey, may interfere with the ability of *a*−*g′* interactions to influence coiled-coil stability in this system. Presumably such effects, exerted by specific sequence contexts, are eliminated by the approach of Keating et al.[10]

We have described a new, small parallel coiled-coil model system and shown how thiol−thioester exchange measurements can provide insights on sequence−stability relationships for this common structural motif. Our system has been validated by reproducing quantitative trends among *a*−*a′* pairing preferences in the coiled-coil core that were previously measured in a substantially larger system,[3a] and by detecting a specifying role for *a*−*g′* ion pairs that was recently suggested based on computational and combinatorial design.[10] We have extended the understanding of Coulombic contributions to coiled-coil stability by showing that such *a*−*g′* pairings can influence coiled-coil pairing specificity, at least for Arg-Glu combinations. From a methodological perspective, it is noteworthy that the thiol component in the new system presented here (e.g., in $_{HS}$**C′**) is provided by a Cys side chain rather than a backbone-mimetic α-thioacid. This design was necessitated by the parallel alignment of helical segments in the structure under study; the success of this approach suggests that the thiol−thioester exchange technique need not be limited to peptidic backbones. The system introduced here should be useful for further delineation of the factors that control parallel coiled-coil stability and pairing selectivity.

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Supporting Information Available: Experimental procedures, CD and AUC data, HPLC chromatograms, and representative structures uncovered using CC+ are included and discussed. This material is available free of charge via the Internet at http://pubs.acs.org.

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