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Heterotrimeric Coiled Coils with Core Residue Urea Side Chains†

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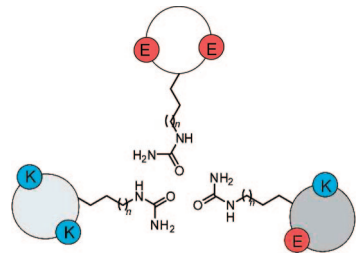

We report several coiled coil heterotrimers with varying core residue buried polar groups, all with T_m values >43 °C. Introduction of new synthetic side chain structures, including some terminating in monosubstituted ureas, diversifies the pool of viable core residue candidates. A study of core charge pairings demonstrates that, unlike dimeric systems, trimeric coiled coils do not tolerate guanidine−guanidine contacts, even in the presence of a compensating carboxylate. Overall, the roster of feasible coiled coil designs is significantly expanded.

Design of self-assembling molecular systems is an efficient way to harness synthetically accessible structures for the creation of otherwise dauntingly sophisticated complexes. Natural systems provide considerable inspiration in this effort, validating particular structural motifs and building blocks and identifying frameworks for advanced development. Systems derived from biological examples also serve to illuminate new and fundamental principles of the underlying molecular interactions, which in turn can be used to close the cycle by perturbation of the blueprint biological systems.[1]

Formation of α helical coiled coils by the supercoiling of two or more component peptide strands is one such motif. Coiled coil recognition governs myriad cellular events ranging from gene expression to membrane fusion.[2] Its ubiquitous biological function places a premium on a detailed understanding of coiled coil formation and stability, while the capacity for programmed assembly of discrete nanoscale structures could facilitate advances in broader arenas.

The basic sequence/structure connections in coiled coils are now well understood: seven residue blocks or heptads (labeled *abcdefg*) are observed, in which each heptad position has a defined structural role. The primary recognition interface is a hydrophobic core comprising *a*/*d* position side chains, with additional interactions observed between *e*/*g* residues on adjacent helices. These principles have been leveraged for production of many synthetic coiled coils, aimed at both elucidating fundamental interactions and applying them to more complex systems.[3] To facilitate next-generation designs, we have been interested in expanding the horizon of compatible interactions at both interfaces. Incorporation of unnatural side chain structures holds promise as a solution to otherwise difficult assembly challenges.

We have recently shown that derivatization of *a*/*d* or *e*/*g* structures in dimeric systems can produce well-defined coiled coils with impressive specificities (Figure 1).[4] In particular, we demonstrated that guanidine and urea-terminated side chains inserted into a single core position were compatible with dimeric structures and, in some cases, were capable of mutual recognition without disturbing other contacts. Here we expand this work to trimeric structures and delineate the stability profiles of numerous new interactions. A number of new core residue alignments are shown to be compatible with trimer formation (each with T_m > 43 °C), further expanding the design toolbox

† Dedicated in memory of the late Professor Albert I. Meyers, who enriched the lives of all he met.

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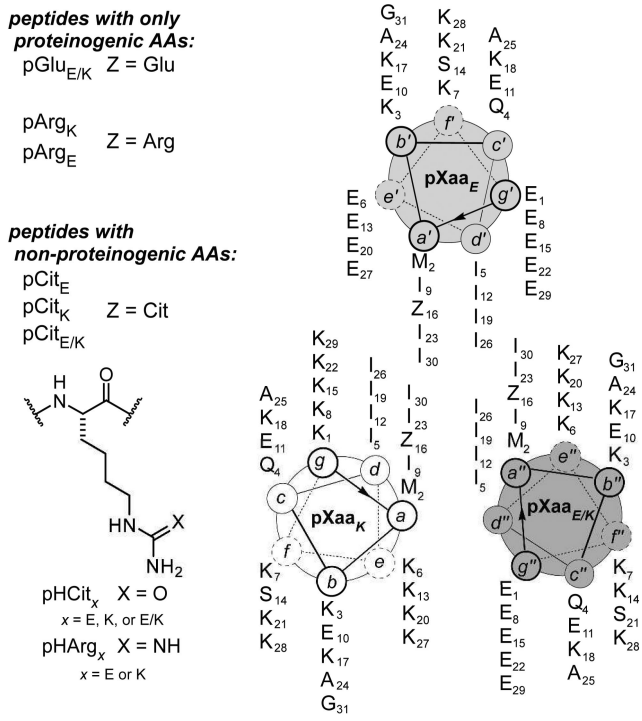
for such structures. In addition, some polar residue combinations expected to be viable based on our dimeric work are demonstrated to be significantly destabilizing.

To evaluate polar core contacts in trimeric systems, we began with sequences we have previously reported, which form a specific 1:1:1 heterotrimer via core residue steric matching.[5] Replacement of most core residues with the trimer-compatible isoleucine residue provided a test scaffold, in which a central core *a* position could be replaced with the polar side chain of choice. The three sequences are distinguished by incorporation of acidic ($pXaa_E$), basic ($pXaa_K$), or mixed ($pXaa_{E/K}$) residues in the core-flanking *e*/*g* positions, which provides electrostatic specificity for heterotrimer formation. Several derivatives of each sequence were prepared by replacing Ile_{16} with the side chain to be evaluated. With an eye toward expansion of side chain diversity, we employed both proteinogenic (Arg, Glu) and nonproteinogenic (citrulline, homocitrullinie, homoarginine) polar side chains. We had not used homocitrulline or homoarginine in our study of dimeric systems but reasoned that the larger trimer cavity might benefit from additional spacing between terminus and backbone. We were also confident that our recently developed methods for on-resin installation of urea or guanidine groups during solid phase peptide synthesis would make preparation of these new structures trivial.[4b,6] Indeed, peptide synthesis proved uneventful, using standard solid phase methods.

Since our central concern was expansion of coiled coil compatible polar contacts, we first investigated systems with all-urea cores, as such structures have not previously been used in trimeric systems. Thus our initial targets were the $pCit_K$/$pCit_{E/K}$/$pCit_E$ and $pHCit_K$/$pHCit_{E/K}$/$pHCit_E$ assemblies. Gratifyingly, circular dichroism (CD) experiments support formation of stable heterotrimers in both cases (Figure 2). Each of the component peptides exhibits little if any secondary structure at room temperature, while the equimolar mixtures display strong α helical signals, consistent with previous coiled coil assemblies.



peptide sequences:

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                 gabcdefgabcdefg a bcdefgabcdefgab
pXaa_E   Ac-EMKQIEKEIEEIESE Z KKIEKEIAAIEKEIG-NH2
pXaa_K   Ac-KMKQIKKIEEIKSK Z KKIKKKIAAIKKKIG-NH2
pXaa_E/K Ac-EMKQIKKEIEEIKSE Z KKIKKEIAAIKKEIG-NH2
                 1    5    10   15   20   25   30
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FIGURE 1. Peptide sequences and helical wheel. Sequences were derived from one of our previously reported heterotrimers by replacement of hydrophobic core residues. They differ in identity of the central polar residue at position 16 and the pattern of electrostatic contacts at *e*/*g* positions.

Only the 1:1:1 mixtures display a cooperative thermal unfolding profile, again consistent with complex formation, and they exhibit reasonable melting temperatures (Table 1).

To investigate trimer stoichiometry, we synthesized $pCit_{KHis}$ by appending a $GlyGly(His)_6$ sequence to the N-terminus of $pCit_K$. The tag confers affinity for nickel nitrilotriaceticacid (Ni-NTA) groups, via the imidazole side chains. This interaction was exploited in an affinity experiment by subjecting an equimolar solution of $pCit_{K\ His}$/$pCit_{E/K}$/$pCit_E$ to Ni-NTA functionalized agarose beads. After supernatant removal and washing, bound material was eluted from the beads with imidazole buffer. HPLC analysis of the elution fractions demonstrated that the tagged peptide retained each binding partner (Figure 3). Similar results were obtained for the homocitrulline trimer, using $pHCit_{KHis}$ to retain 1 equiv of each untagged component.

After validating urea-terminated side chains as buried polar groups compatible with trimer formation, we probed the sensitivity of these interactions to subtle structural modifications. The all-citrulline trimer features ureas spaced from the backbone by nine total methylene groups (three per side chain), while the homocitrulline analogue has 12. Since coiled coil stability is known to be quite sensitive to core residue bulk, intermediate core occupancy was investigated using mixed-core trimers. Equimolar mixtures of $pCit_K$/$pHCit_{E/K}$/$pCit_E$ and $pHCit_K$/$pCit_{E/K}$/$pHCit_E$ provide 10 and 11 methylenes, respectively. Their CD profiles are similarly intermediate (Figure 4), indicating a broad tolerance of side chain size.

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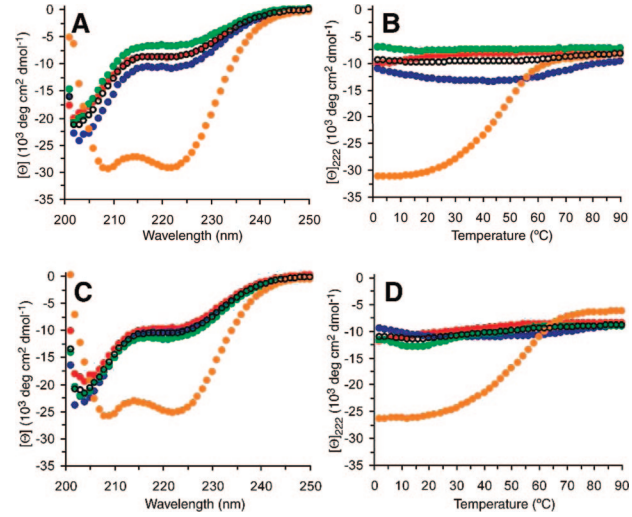

FIGURE 2. Heterotrimers with buried ureas. Wavelength (A, C) and thermal denaturation (B, D) CD data for heterotrimers with citrulline or homocitrulline core residues.[11] Data from pure solutions of component peptides for the citrulline complex (A, B) as follows: $pCit_E$ (red), $pCit_K$ (blue), and $pCit_{E/K}$ (green). For the homocitrulline complex: $pHCit_E$ (red), $pHCit_K$ (blue), and $pHCit_{E/K}$ (green). In all cases, data for the equimolar mixture are in orange, and the calculated component average is in open circles. Significant helicity and stability enhancements for the mixtures over the weighted average signals support trimer formation.

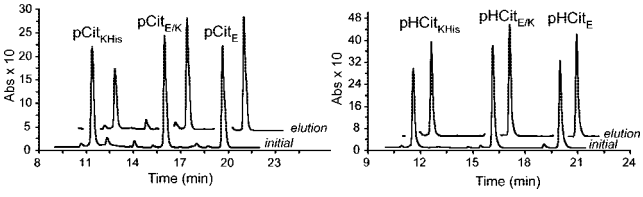

FIGURE 3. Ni-NTA stoichiometry assay. Initial equimolar solutions of $pCit_{KHis}/pCit_{E/K}/pCit_E$ (left) or $pHCit_{KHis}/pHCit_{E/K}/pHCit_E$ (right) were exposed to Ni-NTA agarose beads.[7] After supernatant removal and washing with buffer, bound material was eluted from the beads with imidazole buffer. HPLC traces of the elution fractions indicate retention of untagged peptides, supporting designed stoichiometries.

TABLE 1. Observed T_m Values

sample	T_m (°C)	sample	T_m (°C)
$pCit_K/pCit_{E/K}/pCit_E$	51	$pArg_K/pCit_{E/K}/pCit_E$	47
$pHCit_K/pHCit_{E/K}/pHCit_E$	59	$pArg_K/pHCit_{E/K}/pCit_E$	45
$pCit_K/pHCit_{E/K}/pCit_E$	57	$pCit_K/pCit_{E/K}/pArg_E$	45
$pHCit_K/pCit_{E/K}/pHCit_E$	57	$pCit_K/pCit_{E/K}/pHArg_E$	51
$pArg_K/pGlu_{E/K}/pCit_E$	45	$pCit_K/pGlu_{E/K}/pCit_E$	45
$pHArg_K/pGlu_{E/K}/pHCit_E$	43	$pCit_K/pGlu_{E/K}/pHCit_E$	51
$pHarg_K/pGlu_{E/K}/pCit_E$	45	$pHCit_K/pGlu_{E/K}/pHCit_E$	51

Given this reliable platform, we next investigated the use of charged core side chains, which had provided unique specificity behavior in our dimeric systems. In those prior systems, juxtaposition of a core residue guanidine with other guanidines, ureas, or carboxylates gave rise to stable heterodimeric structures.

Stability of the guanidine/guanidine motif in those cases was unexpected, and we next sought to evaluate its viability in trimeric systems. Using peptides with arginine, homoarginine, citrulline, or homocitrulline at position 16, we prepared equimolar mixtures that could generate trimers with two guanidines and a urea at the central core position. The chain lengths were varied such that the total number of methylene spacers from the backbones ranged from 9 to 12, as in the all-urea examples above. Unlike those systems, however, we were surprised to observe that none of the bisguanidine/urea cores were viable. In each case, CD analysis demonstrated little or no helicity gain for the mixture compared to the component weighted average. Thermal unfolding experiments were similarly unpersuasive, in sharp contrast with our experience in dimeric structures. It is conceivable that the comparatively more solvent-exposed core of dimeric coiled coils is more tolerant of the extra charge.[7]

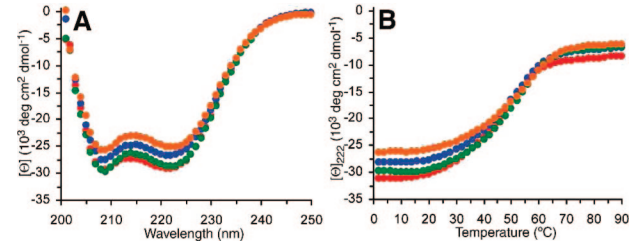

FIGURE 4. Variable length core residues. Wavelength (A) and thermal denaturation (B) CD data for equimolar solutions of $pCit_K/pCit_{E/K}/pCit_E$ (red), $pCit_K/pHCit_{E/K}/pCit_E$ (blue), $pHCit_K/pCit_{E/K}/pHCit_E$ (green), and $pHCit_K/pHCit_{E/K}/pHCit_E$ (orange).[7] Signal similarity indicates flexibility in viable core arrangements.

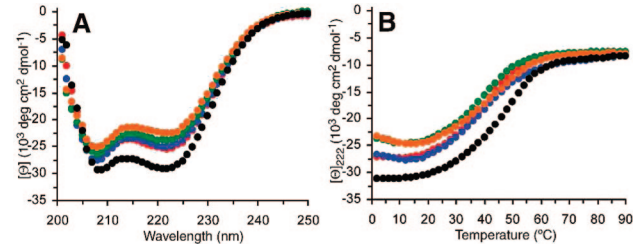

FIGURE 5. Cores with a single positive charge. Wavelength (A) and thermal denaturation (B) CD data for equimolar solutions of $pArg_K/pCit_{E/K}/pCit_E$ (red), $pCit_K/pHCit_{E/K}/pArg_E$ (blue), $pArg_K/pHCit_{E/K}/pCit_E$ (green), and $pCit_K/pCit_{E/K}/pHArg_E$ (orange). The $pCit_K/pCit_{E/K}/pCit_E$ signal (black) is given for reference.[7]

To determine if less demanding charge burial would be tolerated in trimers, we next constructed complexes with a single core guanidine. At the same time, we wondered if any preferences existed for attaching particular polar termini to particular parent scaffolds (e.g., if the guanidine side chain would be better off on the acidic sequence, etc.). We also wondered if some termini would be better off on longer side chains than others.

To examine these questions, we prepared a series of equimolar mixtures. Two contained nine total methylene spacers and differ in attachment of arginine to the acidic or basic parent sequence ($pArg_K/pCit_{E/K}/pCit_E$ and $pArg_K/pCit_{E/K}/pCit_E$). Another pair contained 10 total methylenes and moved the guanidine both from acidic to basic parent and from a four carbon to a three carbon spacer ($pArg_K/pCit_{E/K}/pCit_E$ and $pArg_K/pCit_{E/K}/pCit_E$). In both of these cases, CD data support formation of helical and thermally robust structures (Figure 5). Signals for each pair are essentially identical, another departure from our dimeric work, in which parent sequence identity was often significant. The origins of this effect are not obvious, but it reinforces the results above that suggest a significant difference between dimeric and trimeric core environments.

Since these single guanidine cores were so much more compatible with trimer formation, we wondered if introducing an anionic side chain into the double guanidine systems would

(7) All solutions are 10 μM total peptide concentration in PBS buffer (150 mM NaCl, 10 mM phosphate, pH 7.0).

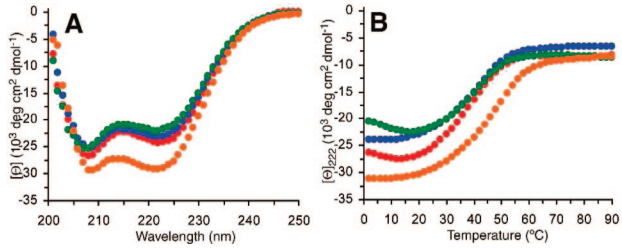

FIGURE 6. Cores with balanced charges. Wavelength (A) and thermal denaturation (B) CD data for equimolar solutions of $pArg_K/pGlu_{E/K}/pCit_E$ (red), $pHArg_K/pGlu_{E/K}/pCit_E$ (blue), and $pHArg_K/pGlu_{E/K}/pHCit_E$ (green). The $pCit_K/pCit_{E/K}/pCit_E$ signal (orange) is given for reference.[7]

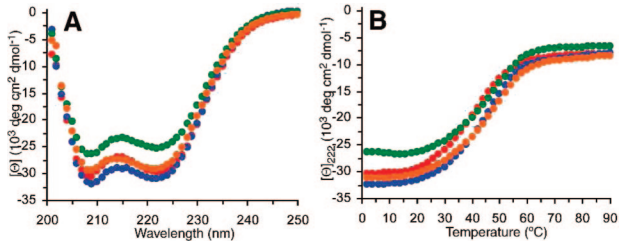

FIGURE 7. Cores with one negative charge. Wavelength (A) and thermal denaturation (B) CD data for equimolar solutions of $pCit_K/pGlu_{E/K}/pCit_E$ (red), $pCit_K/pGlu_{E/K}/pHCit_E$ (blue), and $pHCit_K/pGlu_{E/K}/pHCit_E$ (green). The $pCit_K/pCit_{E/K}/pCit_E$ signal (orange) is given for reference.[7]

produce reasonable complexes with a net single positive charge. We thus investigated several mixtures using $pGlu_{E/K}$ in combination with various guanidine peptides. In each case, however, CD data were at best feebly supportive of trimer formation.

To determine if guanidine/carboxylate pairs were reasonable in any trimeric context, as they had been in our previous dimeric work, we investigated several complexes with net neutral cores. Equimolar mixtures of $pArg_K/pGlu_{E/K}/pCit_E$, $pHArg_K/pGlu_{E/K}/pCit_E$, and $pHArg_K/pGlu_{E/K}/pHCit_E$ all exhibit CD profiles consistent with trimer formation (Figure 6). The variance in total core methylene count seems relatively insignificant for overall stability, as in the other trimeric arrangements.

Given success with the guanidine/carboxylate interaction, we were curious about the behavior of singly anionic cores alone. Mixtures of $pCit_K/pGlu_{E/K}/pCit_E$, $pCit_K/pGlu_{E/K}/pHCit_E$, and $pHCit_K/pGlu_{E/K}/pHCit_E$ all exhibit CD profiles consistent with trimer formation and comparable to each other (Figure 7). They appear slightly more helical and stable than the monocation or cation/anion pairs, though the effect is sufficiently subtle that detailed conclusions are probably unwarranted.

The coiled coil heterotrimers described here are the first to incorporate urea-functionalized side chains as buried polar groups, adding to the structural diversity of reasonable designs. Significantly different behavior was observed in these systems than in analogous heterodimeric structures with similar core side chains, both in terms of viable structural motifs and sensitivity to side chain manipulation.

Though several guanidine/guanidine contacts are feasible in dimers, all such combinations were unsuccessful here, as were those with two positive and one negative side chain. Either guanidine or carboxylate side chains by themselves were tolerated when combined with two ureas, and the inclusion of all three to form net neutral cores was also successful.

Unlike dimeric coiled coils, the trimers reported here seem largely indifferent to specific details of core occupancy as measured by total methylene separation from respective backbones. Similarly, whereas identity of parent sequence was in some cases quite significant for the dimers, these systems give comparable stabilities regardless.

Overall, we have demonstrated that urea-derived side chains are reasonable candidates for the role of buried polar group in coiled coil trimers. Facile synthetic access to new structural motifs allows for diversification of the design toolbox. In turn, this permits fine-tuning of complex stability and raises the possibility of specific orthogonal recognition. The considerable biological significance of coiled coils adds to the motivation for understanding and manipulating these recognition events.

Experimental Section

Peptide Synthesis. Amino acids were obtained from NovaBiochem (San Diego) or Bachem (Torrance, CA), including Boc-Dap (Fmoc)-OH and Boc-Dab (Fmoc)-OH (both from Bachem). Peptides were prepared according to the in situ neutralization protocol[8] for solid phase peptide synthesis developed by Kent. Each peptide was purified by reverse-phase HPLC (C-18 column, solvent A: 1% CH_3CN in H_2O, 0.1% (v/v) CF_3CO_2H; solvent B: 10% H_2O in CH_3CN, 0.07% (v/v) CF_3CO_2H), and the identity of purified samples was confirmed by electrospray mass spectrometry (Finnegan LCQ-Duo). All peptides are C-terminally amidated and N-terminally acetylated; each contains an acetamidobenzoate group on the side chain nitrogen of a solvent-exposed lysine as a spectroscopic label.

Ni-NTA Affinity Tag Experiments. A 0.5 mL sample of a 50% slurry of Ni-NTA agarose (Qiagen) in an Eppendorf tube was centrifuged for 30 s, followed by removal of the superantant. Peptide solution (1 mL of 20 or 10 μM total peptide, 5 μM each peptide) was added, and the tube was repeatedly inverted for 5 min. All peptide solutions were made up in PBS buffer (150 mM NaCl, 10 mM phosphate, pH 7.0). The sample was centrifuged (30 s), and the supernatant (flow-through fraction) was removed. The procedure was then repeated with 1 mL of buffer (wash fraction) and 1 mL of buffer containing 250 mM imidazole (elution fraction), except that the wash fraction was only agitated for 30 s.[9] Solutions were analyzed by RP-HPLC.

CD Spectroscopy. All experiments were performed on an Aviv model 202 circular dichroism spectrometer, equipped with a Microlab 500 series automated titration assembly. Sample concentrations were measured by UV absorbance of the acetamidobenzoate label at 270 nm. Wavelength data are the average of three scans from 250 to 200 nm in 1 nm steps. Thermal denaturation experiments at 222 nm were run from 0 to 90 °C in two-degree steps, at a two-degrees/minute rate of increase with one-minute equilibration and data averaging at each temperature. T_m values were obtained from minima of the first derivative of θ versus $1/T$ plots.[10]Peptides were dissolved in PBS buffer (150 mM NaCl, 10 mM phosphate, ph 7.0).

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Supporting Information Available: Wavelength and thermal denaturation CD spectra for trimers with two guanidines or two guanidines and an acid in the core. This material is available free of charge via the Internet at http://pubs.acs.org.

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(8) Data for these experiments can be found in the Supporting Information.

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