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# Strand Orientation by Steric Matching: A Designed Antiparallel Coiled-Coil Trimer

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***Abstract:*** The design of an antiparallel coiled-coil 1:1:1 heterotrimer is described. Control of strand orientation results from proper alignment of sterically matched hydrophobic core side chains. Matched core layers position one cyclohexylalanine side chain against two alanine ones. Substitution of three consecutive heptad *a* positions with all permutations of two alanines and one cyclohexylalanine (AAX, AXA, AAX, where A = alanine, X = cyclohexylalanine) affords a parallel 1:1:1 heterotrimer, as previously reported. Here, we report that moving the substitution sites in one strand to *d* rather than *a* positions affords a new peptide that can form an antiparallel complex with the other original components. The new assembly is characterized by circular dichroism spectroscopy ($[\theta]_{222} = -30\,317$ deg cm$^2$ dmol$^{-1}$, $T_m = 77$ °C, $\Delta G_{unf} = 17.1$ kcal/mol), and its stoichiometry and aggregation number are confirmed by nickel tag affinity analysis and analytical ultracentrifugation. Disulfide exchange data support the preference for an antiparallel arrangement. Examination of the functionally identical parallel complex demonstrates that the antiparallel structure is comparably stable, as confirmed by a direct competition assay that established an equilibrium 55:45 ratio of each assembly.

## Introduction

Reliable manipulation of protein−protein recognition would provide manifold new opportunities for therapeutic intervention, as virtually all biological events are dependent upon such contacts. Model systems for common assembly motifs can establish the foundation for such achievement through illumination of critical structural requirements. The α-helical coiled-coil, comprised of two or more helical strands entwined by a superhelical twist, is one such bedrock motif.[1] Ubiquitous cellular application and relative structural simplicity render it an attractive molecular design target.[2]

Early coiled-coil investigations established the prevalence of a primary sequence heptad repeat (*abcdefg*), in which hydrophobic residues at *a* and *d* positions are key determinants of complex stability and assembly preferences. Additional specificity is conferred by hydrophilic *e*/*g* side chains, whose polar termini allow for electrostatic recognition. These features have been exploited in many model complexes, and much is known about their manipulation. Most of these efforts have focused on parallel complexes, in which the N- and C-termini of all strands are aligned.

Antiparallel structures have proven more challenging, in part because isolated native sequences are less well-behaved than their parallel counterparts.[3] Nonetheless, driven in part by a growing appreciation for their previously underestimated biological importance, clever designs of several antiparallel systems have been reported.[4−6] All take advantage of differential side-chain packing between parallel and antiparallel orientations (Figure 1). In parallel complexes, the hydrophobic core is comprised of alternating layers of *a* and *d* side chains, while interhelical electrostatic contacts are possible between *e* and *g* residues. Antiparallel structures exhibit mixed *a*/*d* core layers and three distinct hydrophilic interfaces (*e*/*g*, *e*/*e*, and *g*/*g*). Thus, careful choice of side-chain patterning can promote the desired orientation. Burial of a single core asparagine favors alignments that permit Asn−Asn packing, rather than polar/nonpolar contacts.[4] Steric matching of core side chains (e.g., Ile/Ala, Leu/Ala) can also influence parallel/antiparallel preferences.[5] In most cases, these core modifications are combined with *e*/*g* sequences

(1) Reviews: (a) Burkhard, P.; Strelkov, S. V.; Stetefeld, J. *Trends Cell Biol.* **2001**, *11*, 82−88. (b) Lupas, A. *Trends Biochem. Sci.* **1996**, *21*, 375−382.
(2) Reviews: (a) Micklatcher, C.; Chmielewski, J. *Curr. Opin. Chem. Biol.* **1999**, *3*, 724−729. (b) DeGrado, W. F.; Summa, C. M.; Pavone, V.; Nastri, F.; Lombardi, A. *Annu. Rev. Biochem.* **1999**, *68*, 779−819 and references therein.
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(5) (a) Gurnon, D. G.; Whitaker, J. A.; Oakley, M. G. *J. Am. Chem. Soc.* **2003**, *125*, 7518−7519. (b) Lombardi, A.; Bryson, J. W.; DeGrado, W. F. *Biopolymers* **1996**, *40*, 495−504. (c) Betz, S. F.; DeGrado, W. F. *Biochemistry* **1996**, *35*, 6955−6962. (d) Monera, O. D.; Zhou, N. E.; Lavigne, P.; Kay, C. M.; Hodges, R. S. *J. Biol. Chem.* **1996**, *271*, 3995−4001. (e) Monera, O. D.; Kay, C. M.; Hodges, R. S. *Biochemistry* **1994**, *33*, 3862−3871. (f) Lovejoy, B.; Choe, S.; Casico, D.; McRorie, D. K.; DeGrado, W. F.; Eisenberg, D. *Science* **1993**, *259*, 1288−1293. (g) Monera, O. D.; Zhou, N. E.; Kay, C. M.; Hodges, R. S. *J. Biol. Chem.* **1993**, *268*, 19218−19227.
(6) Two notable exceptions are the serendipitous crystallization of an antiparallel trimer with apparently mismatched electrostatic interfaces (ref 5f), and the formation of an $A_2B_2$ tetramer with identical interactions in either orientation (ref 5d).

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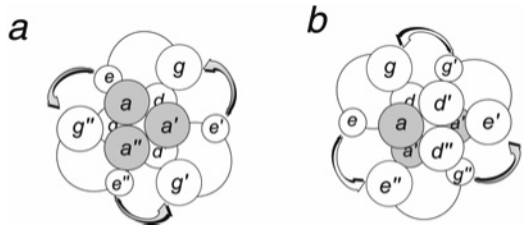


***Figure 1.*** Differential core organizations in trimeric coiled-coils. Schematic view of a single heptad emphasizes the separated (*a*, *a*′, *a*″ and *d*, *d*′, *d*″) or mixed (*a*, *d*′, *d*″ and *a*′, *a*″, *d*) hydrophobic core layers exhibited by parallel (a) and antiparallel (b) structures, respectively.

that align optimal electrostatic contacts (e.g., matched Glu/Lys pairs) only in the preferred complex.[6]

We have previously demonstrated that steric matching of 2:1 alanine:cyclohexylalanine core layers at three consecutive *a* positions ensures specific assembly of parallel heterotrimers.[7] We therefore reasoned that pairing two *a* substituted sequences with one bearing alanine/cyclohexylalanine residues at *d* positions should favor an antiparallel assembly. In particular, we sought to simultaneously control stoichiometry and strand orientation in a coiled-coil trimer using only core steric matching. The paucity of antiparallel trimeric designs, coupled with the importance of electrostatic specificity in other systems, made this a significant challenge. Here, we describe the application of our prior methods to the construction of an antiparallel 1:1:1 heterotrimer. It displays circular dichroism (CD) behavior consistent with a well-formed coiled-coil, and its stoichiometry and aggregation state are as expected. The preference for antiparallel strand orientation is convincingly demonstrated by a disulfide exchange assay, and direct comparisons with the analogous parallel complex reveal that it is of comparable stability.

## Results and Discussion

Design of the antiparallel complex began with the known $T_9K$:$T_{16}E$:$T_{23}E/K$ heterotrimer.[8] This parent assembly contains sterically matched central core *a* layers of XAA, AXA, and AAX (X = cyclohexylalanine), and three electrostatically matched *e*/*g* interfaces (all pairing Glu/Lys). Replacement of $T_{16}E$ with $T_{16}E^a$, bearing the AXA sequence at consecutive *d* positions, affords a trimer that must adopt an antiparallel orientation to properly align its core side chains, and to bury the optimal number of core residues (vide infra). Because Glu is present at all *e*/*g* positions of both $T_{16}E$ and $T_{16}E^a$, the strand flip does not alter any electrostatic contacts, thus limiting the means to control orientation to core interactions (Figure 2).

Initial feasibility of the designed antiparallel system was demonstrated by CD, and in particular by comparison with the parallel behavior (Figure 3). The wavelength spectra of 1:1:1 $T_9K$:$T_{16}E^a$:$T_{23}E/K$ (antiparallel) and 1:1:1 $T_9K$:$T_{16}E$:$T_{23}E/K$ (parallel) solutions are quite similar ($[\theta]_{222}$ = −30 317 vs −32 798 deg cm$^2$ dmol$^{-1}$), as are the thermal unfolding profiles

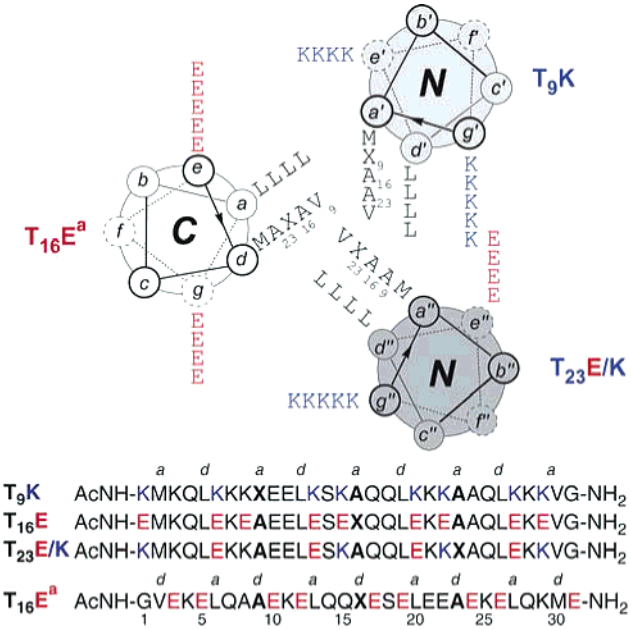





***Figure 2.*** Helical wheel projection of designed complex and sequences employed. Nomenclature is of the form $T_nZ$ (derived from previously reported[5a] peptides $T_9$, $T_{16}$, $T_{23}$), where *n* indicates the cyclohexylalanine position and Z reflects content of all *e*/*g* residues. In $T_{16}E^a$, core substitutions occur at *d* (rather than *a*) heptad positions. Wheel projection looks down on the N-termini of $T_9K$ and $T_{23}E/K$, and the C-terminus of $T_{16}E^a$.

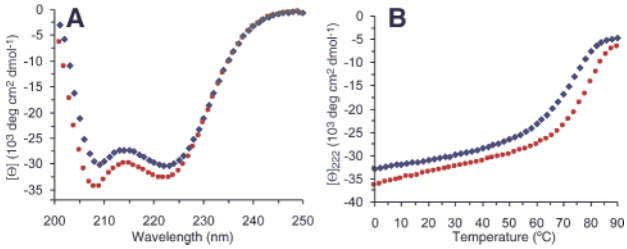


***Figure 3.*** Wavelength (A) and thermal denaturation (B) circular dichroism data for solutions of $T_9K$:$T_{16}E$:$T_{23}E/K$ (red ●) and $T_9K$:$T_{16}E^a$:$T_{23}E/K$ (blue ◆). All solutions are 10 $\mu$M total peptide in PBS buffer (10 mM phosphate, pH 7.5, 150 mM NaCl). Wavelength spectra were taken at 25 °C.

and melting temperatures ($T_m$ = 77 vs 81 °C). Unfolding free energies measured by GdnHCl denaturation are also comparable ($\Delta G_{unf}$ = 17.1 vs 18.2 kcal/mol).[9]

Verification of complex stoichiometry was executed using a previously described affinity assay. A derivative of $T_{23}E/K$ bearing an N-terminal Gly−Gly−$(His)_6$ tag sequence ($T_{23}E/K_{His}$) was mixed with $T_9K$ and $T_{16}E^a$ in a 1:1:1 ratio. The solution was exposed to nickel−nitrilotriacetic acid (Ni−NTA) agarose beads, followed by washing to prevent artifacts from nonspecific binding and elution with imidazole buffer to recover bound material. HPLC analysis demonstrated retention of each untagged peptide, confirming their affinity for $T_{23}E/K$.[9] Independent support for trimer formation was obtained from analytical ultracentrifugation ($M_{rcalc}$ = 11 563, $M_{robs}$ = 11 933).[9]

Having established the formation of a well-behaved coiled-coil, we conducted a disulfide exchange experiment to support the designed antiparallel strand arrangement (Figure 4).[10] Required peptides were prepared by separate attachment of Gly−Gly−Cys sequences to the N-terminus of $T_9K$ ($T_9K_{N-Cys}$)

(7) (a) Schnarr, N. A.; Kennan, A. J. *J. Am. Chem. Soc.* **2002**, *124*, 9779−9783. (b) Schnarr, N. A.; Kennan, A. J. *J. Am. Chem. Soc.* **2003**, *125*, 667−671.

(8) Although this complex is technically new, its behavior should mirror the functionally identical $T_9K$:$T_{16}E/K$:$T_{23}E$ trimer reported previously (ref 7b). Subscripts indicate position of cyclohexylalanine; letters correspond to amino acids in *e*/*g* positions. Note that the leading "T" in the peptide names does not stand for threonine, but refers to the tic-tac-toe-like arrangement of side chains at the critical core layers (for a detailed genesis of the nomenclature, see ref 5a).

(9) See the Supporting Information for additional details.

(10) See refs 4b,d for previous applications of this method.

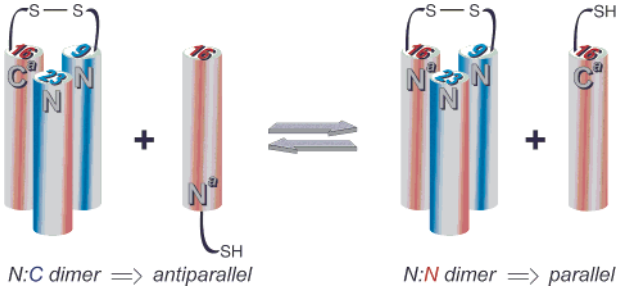


***Figure 4.*** Disulfide exchange orientation assay. A mixture of $T_{23}E/K$ with thiol containing derivatives of $T_9K$ and $T_{16}E^a$ (Gly−Gly−Cys at the N-terminus and C-terminus, respectively) permits disulfide formation in the antiparallel complex (left). Treatment with an N-terminally modified $T_{16}E^a$ permits exchange to a new disulfide in a parallel complex (right). Equilibrium populations of respective disulfides indicate orientation preference. Red and blue stripes indicate glutamic acid and lysine, respectively, at *e*/*g* interfaces. Note that helix representations are schematic. In particular, the undesired parallel complex is likely out of register (see text).

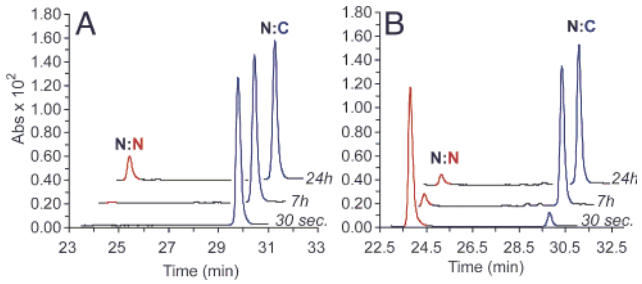


***Figure 5.*** Disulfide exchange data for the antiparallel $T_9K:T_{16}E^a:T_{23}E/K$ complex (see Figure 4). HPLC traces of the disulfide region taken after indicated intervals.[9] (A) Initial N:C disulfide remains largely unchanged. (B) Initial N:N disulfide rearranges to N:C species which can form the favored antiparallel complex.

and to either end of $T_{16}E^a$ ($T_{16}E^a{}_{N-Cys}$ and $T_{16}E^a{}_{C-Cys}$). Because the N-terminus of $T_9K$ and C-terminus of $T_{16}E^a$ are proximal in the antiparallel structure, disulfide formation between peptides bearing thiols at those positions (an N:C dimer) should be facile, if they align as intended. In contrast, intracomplex bonding between $T_9K_{N-Cys}$ and $T_{16}E^a{}_{N-Cys}$ (an N:N dimer) requires that they be oriented parallel to each other. Given direct competition between the N:C and N:N dimers, relative disulfide populations reveal the preferred orientation: predominance of the N:C dimer should be observed when the antiparallel complex is favored, while the opposite outcome (more of the N:N dimer) would reflect an equilibrium shifted toward the parallel trimer.

The exchange equilibration was conducted from two different starting points. A mixture of preformed $T_9K_{N-Cys}/T_{16}E^a{}_{C-Cys}$ disulfide (N:C dimer) with $T_{23}E/K$ and $T_{16}E^a{}_{N-Cys}$ (1:1:1 ratio) remains largely unchanged over 24 h (Figure 5a). The lack of N:N dimer formation is consistent with the designed antiparallel arrangement. Perhaps more significantly, when the experiment is run by preforming the N:N dimer between $T_9K_{N-Cys}$ and $T_{16}E^a{}_{N-Cys}$, near complete rearrangement to the N:C dimer is observed (Figure 5b). The similarity of the observed final ratios argues strongly in favor of the antiparallel trimer.

To rule out bias from intrinsic differences in disulfide stability, an analogous series of experiments was conducted on the parent $T_9K:T_{16}E:T_{23}E/K$ heterotrimer. Because this complex can form a parallel trimer with sterically matched core side chains, the exchange assay results should reverse (i.e., N:N dimer dominates) if the experiment is a true reporter of orientation preference. As expected, an equimolar mixture of $T_9K_{N-Cys}/$

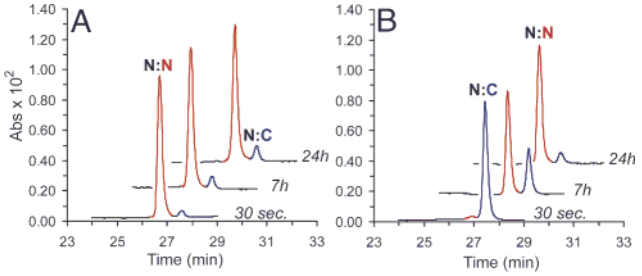


***Figure 6.*** Disulfide exchange data for the parallel $T_9K:T_{16}E:T_{23}E/K$ complex. HPLC traces of the disulfide region taken after indicated intervals.[9] (A) Initial N:N disulfide remains largely unchanged. (B) Initial N:C disulfide rearranges to N:N species which can form the favored parallel orientation.

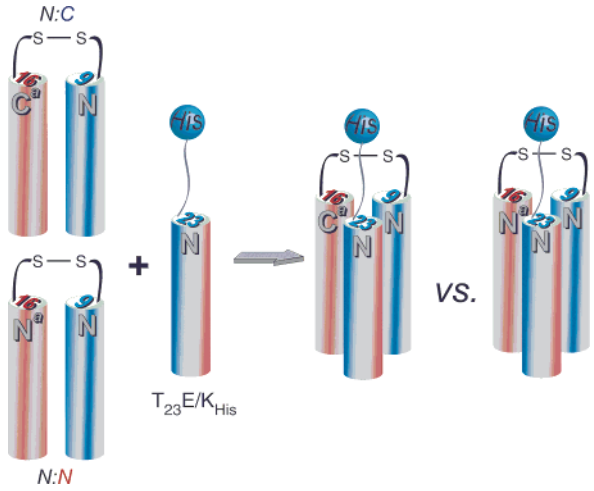


***Figure 7.*** Schematic of a combined disulfide/Ni-affinity competition assay. Preformed disulfides between $T_9K_{N-Cys}:T_{16}E^a{}_{C-Cys}$ (N:C) and $T_9K_{N-Cys}:T_{16}E^a{}_{N-Cys}$ (N:N) can form antiparallel or parallel complexes, respectively, with $T_{23}E/K_{His}$. Analysis of the elution fraction from an initial equimolar mixture reveals relative orientation preference. As in Figure 4, the helix representations should be considered schematic.

$T_{16}E_{N-Cys}$ disulfide (N:N) and $T_{23}E/K$ is virtually exchange inert over 24 h (Figure 6a). In contrast, the preformed N:C disulfide ($T_9K_{N-Cys}/T_{16}E_{C-Cys}$) rearranges to the now preferred N:N dimer (Figure 6b). Thus, disulfide equilibration can accurately report trimer orientation independent of the absolute preference for parallel or antiparallel assemblies.

Although the disulfide exchange data are likely sufficient to conclude that the designed $T_9K:T_{16}E^a:T_{23}E/K$ complex is antiparallel, further verification of assembly orientation was obtained by use of the preformed disulfides in a Ni-affinity experiment. The N:C and N:N disulfides of $T_9K$ and $T_{16}E^a$ (as described above) were mixed in an equimolar ratio with $T_{23}E/K_{His}$, without any free thiol to initiate equilibration. Upon exposure to Ni−NTA beads, the two disulfides must compete for the tagged peptide to be retained. Thus, their relative populations in the elution fraction should reflect the preference for parallel versus antiparallel complexes (Figure 7). In this case, HPLC traces of the supernatant and elution fractions display the expected compositions: the former contains virtually all of the N:N dimer, while the N:C species dominates the latter (Figure 8). The selection of essentially only that disulfide which can form the designed complex is further support of its viability.

The above experiments provide convincing evidence that a 1:1:1 $T_9K:T_{16}E^a:T_{23}E/K$ mixture forms well-behaved antiparallel coiled-coil trimers. Because electrostatic interactions are identical in both the parallel and the antiparallel complexes, the

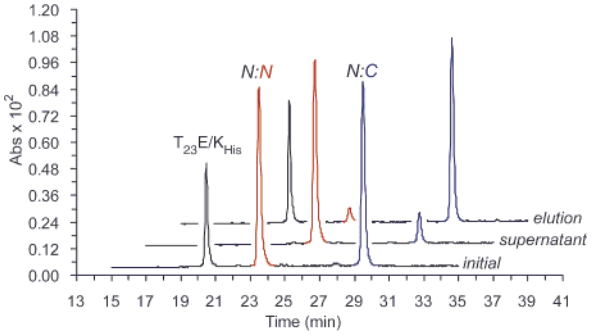


***Figure 8.*** Disulfide/Ni-affinity competition data for the designed complex. The initial solution is equimolar in $T_{23}E/K_{His}$, $T_9K_{N-Cys}$:$T_{16}E^a_{C-Cys}$ (N:C), and $T_9K_{N-Cys}$:$T_{16}E^a_{N-Cys}$ (N:N). After exposure to Ni−NTA agarose beads, virtually all of the N:N dimer remains in the supernatant, while the N:C disulfide appears in the elution fraction, supporting the expected antiparallel preference. See Figure 7 for the schematic representation.

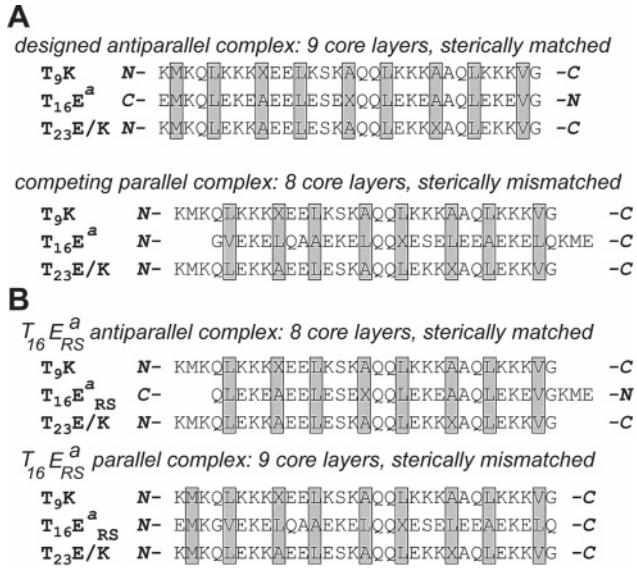


***Figure 9.*** Differential core formation. (A) The designed $T_9K$:$T_{16}E^a$:$T_{23}E/K$ complex can form nine sterically matched core layers in an antiparallel orientation, but only eight mismatched layers in the parallel one. (B) The control $T_9K$:$T_{16}E^a_{RS}$:$T_{23}E/K$ complex must choose between an antiparallel eight matched layer arrangement, and a parallel nine mismatched layer one. Thus, the control sequence determines whether the number or type of core layers is most significant for controlling strand orientation. The core interactions are boxed.

controlling influence in orientation preference must be hydrophobic core interactions. Although we were confident that steric matching was predominant among these, the particular sequences involved also require that the disfavored parallel structure be somewhat register shifted to match core side chains at all (Figure 9). As a result, it can form only eight hydrophobic core layers (in contrast with nine in the antiparallel orientation). To determine if the reduction in total core interactions was critical to the choice of antiparallel arrangement, we relied again on the disulfide/Ni−NTA assay.

A new peptide was prepared ($T_{16}E^a_{RS}$), such that when combined with $T_9K$ and $T_{23}E/K$ it can form either an antiparallel complex with sterically matched core side chains and an equivalent register shift to that above, or a parallel complex that is fully in register but has a sterically mismatched core (Figure 9). Formation of the $T_9K_{N-Cys}/T_{16}E^a_{RS-C-Cys}$ (N:$C_{RS}$) and $T_9K_{N-Cys}/T_{16}E^a_{RS-N-Cys}$ (N:$N_{RS}$) disulfides, followed by purification, equimolar mixing with $T_{23}E/K_{His}$, and exposure to

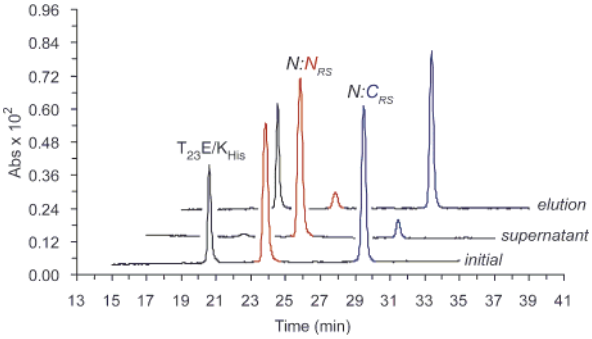


***Figure 10.*** Disulfide/Ni-affinity competition data for the registered shift sequence. The initial solution is equimolar in $T_{23}E/K_{His}$, $T_9K_{N-Cys}$:$T_{16}E^a_{RS-C-Cys}$ (N:$C_{RS}$), and $T_9K_{N-Cys}$:$T_{16}E^a_{RS-N-Cys}$ (N:$N_{RS}$). After exposure to Ni−NTA agarose beads, virtually all of the N:$N_{RS}$ dimer remains in the supernatant, while the N:$C_{RS}$ disulfide appears in the elution fraction, supporting the expected antiparallel preference. See Figure 7 for the schematic representation.

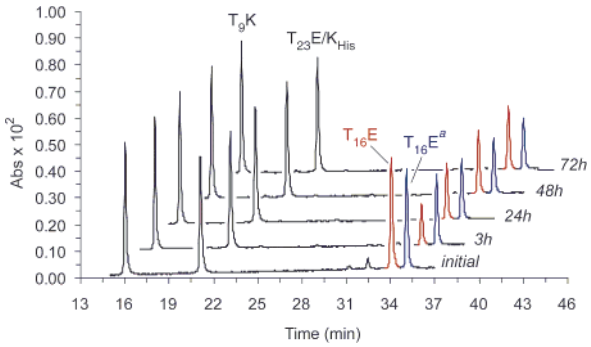


***Figure 11.*** Direct parallel/antiparallel competition. The front HPLC trace shows the initial 1:1:1:1 $T_9K$:$T_{16}E$:$T_{16}E^a$:$T_{23}E/K_{His}$ mixture. The remaining traces show elution fractions after indicated times. The $T_{16}E$:$T_{16}E^a$ peak ratio (for numerical values, see the Supporting Information) corresponds to the relative population of $T_9K$:$T_{16}E$:$T_{23}E/K_{His}$ and $T_9K$:$T_{16}E^a$:$T_{23}E/K_{His}$ heterotrimers.

Ni−NTA beads, produced the desired result (Figure 10). As before, the antiparallel-compatible dimer (N:C) was retained by $T_{23}E/K_{His}$, indicating that steric matching is preferred even at the cost of register shifting. Data from wavelength and thermal denaturation CD experiments on the unfunctionalized $T_9K$:$T_{16}E^a_{RS}$:$T_{23}E/K$ trimer are consistent with those observed for the designed antiparallel complex ($[\theta]_{222} = -30\,317$ vs $-29\,272$ deg cm$^2$ dmol$^{-1}$, $T_m = 77$ vs 71 °C for antiparallel and register shifted complexes, respectively).[9]

Having established a successful designed antiparallel trimer, we returned to the issue of its relation to the parallel parent complex. Both have identical fully matched hydrophobic cores and electrostatic interfaces, but potentially differ in subtle interactions (e.g., macroscopic dipoles, specific side-chain packing angles). Their respective CD behavior (above) suggests that such differences do not dramatically influence their relative stability, which should be confirmable by means of a direct competition experiment employing the Ni−NTA method. A 1:1:1:1 $T_9K$:$T_{16}E$:$T_{16}E^a$:$T_{23}E/K_{His}$ mixture establishes such a competition, in which the $T_9K$ and $T_{23}E/K_{His}$ peptides can bind $T_{16}E^a$ to form the antiparallel complex or $T_{16}E$ to form the parallel one. Elution fractions taken at various time intervals reveal an equilibrium $T_{16}E$:$T_{16}E^a$ peak ratio only slightly in favor of the parallel complex (55:45), in keeping with the above data (Figure 11).

## Conclusions

Taken together, these data document the successful design of an antiparallel trimer, using steric matching of core side chains to control strand orientation. Wavelength and denaturation CD profiles are consistent with a well-formed coiled-coil, while Ni−NTA and analytical ultracentrifugation experiments confirm complex stoichiometry and aggregation number. The preference for antiparallel orientation is established by independent disulfide exchange and Ni−NTA assays. A direct examination of steric matching versus optimal heptad burial reveals that matched cores are formed even at the expense of a single core layer, further underlining the power of this strategy. The designed complex compares favorably in all respects with the analogous parallel one, including direct competition for a limiting binding partner. Given the growing significance of native antiparallel constructs, the capacity for straightforward construction of such antiparallel model systems should be of great value in evaluating critical interaction hypotheses.

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**Supporting Information Available:** Detailed experimental procedures, Ni−NTA, disulfide exchange, analytical ultracentrifugation, and guanidine titration data. CD analysis of register shifted complex. This material is available free of charge via the Internet at http://pubs.acs.org.

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