

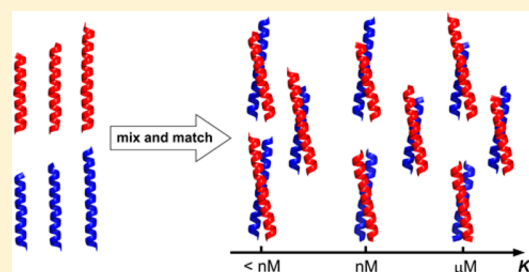
A Set of *de Novo* Designed Parallel Heterodimeric Coiled Coils with Quantified Dissociation Constants in the Micromolar to Sub-nanomolar Regime

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S Supporting Information

ABSTRACT: The availability of peptide and protein components that fold to defined structures with tailored stabilities would facilitate rational protein engineering and synthetic biology. We have begun to generate a toolkit of such components based on *de novo* designed coiled-coil peptides that mediate protein–protein interactions. Here, we present a set of coiled-coil heterodimers to add to the toolkit. The lengths of the coiled-coil regions are 21, 24, or 28 residues, which deliver dissociation constants in the micromolar to sub-nanomolar range. In addition, comparison of two related series of peptides highlights the need for including polar residues within the hydrophobic interfaces, both to specify the dimer state over alternatives and to fine-tune the dissociation constants.



■ INTRODUCTION

Many natural proteins fold efficiently into defined three-dimensional structures that determine their functions. This has inspired studies to probe sequence-to-structure relationships in proteins, and to apply the resulting understanding in the *de novo* design of peptides and proteins. In both respects, the α -helical coiled coil is among the best-understood peptide/protein folds, and one of the most straightforward protein–protein interaction motifs. These attributes have made coiled coils key targets for rational protein design and assembly.^{1,2}

Most coiled-coil sequences have heptad patterns of hydrophobic (h) and polar (p) residues, hpphppp, often denoted as *abcdefg*. These direct the folding of amphipathic helices, which associate through their hydrophobic (*a/d*) faces (Figure 1).^{3–5} The helices wrap around each other to form a left-handed superhelix, in which interhelical side chains pack in a characteristic knobs-into-holes manner.⁶ Although these basic

sequence features seem simple, coiled coils exhibit a diversity of structures in terms of oligomerization state and helix orientation: coiled-coil dimers to hexamers as well as higher-order assemblies, parallel and antiparallel orientations, and homo- or heteromeric systems are all known.^{5,7,8}

Many efforts have been made to uncover sequence-to-structure relationships, or “rules for protein folding”, that direct specific assemblies within the coiled-coil structural landscape,^{9–12} and successful *de novo* designs of a broad spectrum of coiled-coil assemblies have been reported so far.^{13–16} We aim to test and adopt rules for folding and assembly in the systematic creation of a toolkit of *de novo* designed coiled coils. In turn, this can be used in a “plug-and-play” fashion in the context of synthetic biology.^{17,18} Key requirements for such a toolkit include that its components are (1) relatively straightforward in sequence and structure, (2) reliably and stably folded, (3) well characterized under conditions appropriate for later applications, and ideally (4) robust to mutation and engineering within different contexts. In this spirit, we have generated a basis set of *de novo* designs for parallel coiled coils, including a homodimer, homotrimers, and a homotetramer, and we have applied these to control oligomerization.^{18,19} To broaden the toolkit, here we target the rational design of heterodimeric coiled coils.

The utility of heterodimeric coiled coils as mediators of protein associations is apparent from the variety of applications presented, which include mimicry of SNARE-proteins in membrane fusion, modulation of signal transduction pathways, design of artificial EGFR-receptors and ultrasmall antibodies,

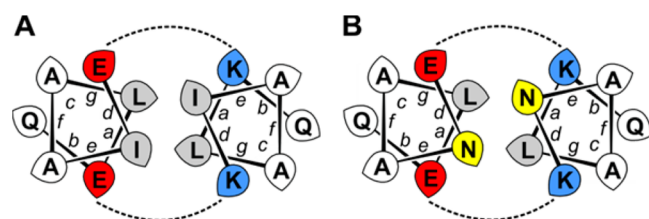


Figure 1. Helical-wheel diagrams showing the coiled-coil heptad repeat. Leaf shapes indicate the direction of the α -C β vectors. Coiled-coil assembly occurs through the *a*, *d*, *e*, and *g* residues. (A) Sequence of the canonical heptad repeats of the CC-A₁B₁ (I-series) designs described herein. (B) Similar sequences for the CC-A₁B₁ (N-series) designs.

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and development of self-assembling biomaterials and discrete nanostructures.^{20–26}

Several design strategies for heterodimeric coiled coils are described in the literature: (1) bottom-up *de novo* design, such as for the E/K-peptides of Hodges et al.;^{27,28} (2) mutation of natural leucine zippers;²⁹ (3) computational refinements of established design rules found empirically;³⁰ and (4) computational analysis combined with experimental combinatorial screens^{31–33} to uncover higher association specificity for the designed peptides.

Although many aspects of heterodimeric coiled-coil designs have been addressed so far, such as making strong coiled-coil interactions²⁹ and ensuring orthogonal assemblies,^{30,32,34} the control, tuning, and quantification of complex stabilities over a range of interaction strengths still requires attention. Coiled-coil stability increases dramatically with peptide chain length, as demonstrated for a set of homodimeric coiled coils with chain lengths varying from one to five heptads.³⁵ We intended to adopt this principle to engineer and quantify a range of stabilities into heterodimeric coiled coils.

MATERIALS AND METHODS

General Methods. Fmoc-protected amino acids, HBTU, and peptide-grade DMF were purchased from AGTC Bioproducts (Hessle, U.K.). The H-Rink Amide-Chemmatrix resin was acquired from PCAS BioMatrix Inc. (Saint-Jean-sur-Richelieu, Canada). All other chemicals were purchased from Fisher Scientific (Loughborough, U.K.). Water was purified with a Synergy UV water purification system from Millipore. Unless otherwise stated, biophysical measurements were performed in phosphate-buffered saline (PBS: 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl). Peptide concentrations were determined by UV absorbance ($\epsilon_{280}(\text{Trp}) = 5690 \text{ mol}^{-1} \text{ cm}^{-1}$, $\epsilon_{280}(\text{Tyr}) = 1280 \text{ mol}^{-1} \text{ cm}^{-1}$) using a NanoDrop 2000 spectrophotometer from Thermo Scientific.

Peptide Synthesis. The *N*-acetylated peptides were synthesized on a H-Rink Amide-Chemmatrix resin on a 0.1 mmol scale on a CEM microwave-assisted synthesizer. The synthesis was conducted via a standard Fmoc protocol using HBTU as coupling reagent. *N*-Acetylation of the peptides was carried out using acetic acid anhydride/pyridine (1:9). Acidic cleavage from the resin was achieved by treatment of the resin with a mixture of trifluoroacetic acid (TFA)/triisopropylsilane/water (90:5:5, 3 h). The resin was extracted with additional TFA (5 mL), and the combined extracts were concentrated to a third of the initial volume under a flow of nitrogen. The crude peptide was then precipitated in cold diethyl ether (40 mL) and isolated by centrifugation and decantation of the ether. The precipitate was redissolved in 5 mL of a 1:1 mixture of acetonitrile/water and then freeze-dried to give a fine white solid.

Peptide Purification. Peptides were purified by reversed-phase HPLC using a Kromatek C18HQsil column (150 by 10 mm). Depending on the peptide sequence, different linear gradients and buffers were used. The basic (B) peptides were purified by running linear gradients of water and acetonitrile (buffer A, water and 0.1% TFA; buffer B, acetonitrile and 0.1% TFA) from 10 to 40% buffer B over 30 min. The acidic (A) peptides were purified by running linear gradients of a triethylammonium acetate buffer (pH 9.5) and basic acetonitrile (buffer C, 20 mM triethylamine and 20 mM acetic acid; buffer D, 99% acetonitrile and 1% buffer C) run from 10 to 40% buffer D over 30 min (CC-A_N) or from 10 to 50% D over 30 min (CC-A_B, peptides modified with cysteine *tert*-butyl ester).

Peptide Characterization. The peptides were characterized by mass spectrometry on an Applied Biosystems 4700 Proteomics Analyzer MALDI-TOF instrument (matrix: α -cyano-4-hydroxycinnamic acid (CHCA), external calibration). Analytical HPLC measurements were performed using a JASCO chromatography system (column with acidic solvent, Phenomenex Prodigy ODS-3, 5 μm , 4.6 \times 100 mm; column with basic solvent, Grace Vydac 214TP C8, 5

μm , 4.6 \times 250 mm). For peptide characterization, acidic (buffers A and B) and basic buffers (buffers C and D) were used.

Circular Dichroism (CD) Spectroscopy. CD spectra and thermal-denaturation curves were recorded using a JASCO J-810 spectropolarimeter fitted with a Peltier temperature controller. CD spectra were measured at 100 μM total peptide concentration in PBS (8.2 mM Na₂HPO₄, 1.8 mM K₂HPO₄, 137 mM NaCl, pH 7.4) at 20 $^{\circ}\text{C}$ in 1 mm (50–300 μM total peptide concentration) and 10 mm (2–20 μM total peptide concentration) quartz cuvettes from STARNA at 50 nm/min scanning speed. Thermal-denaturation experiments were performed by heating from 5 to 90 $^{\circ}\text{C}$ at a rate of 40 $^{\circ}\text{C}/\text{h}$. The CD signal at 222 nm was recorded at 1 $^{\circ}\text{C}$ intervals (1 nm interval, 1 nm bandwidth, 16 s response time). The midpoints of the thermal-denaturation curves (T_m) were determined from the second derivative of the variable-temperature slope. For each experiment, triple measurements were performed. Errors were obtained from standard deviations.

Analytical Ultracentrifugation (AUC). Sedimentation-equilibrium experiments were conducted at 20 $^{\circ}\text{C}$ in a Beckman-Optima XL-I analytical ultracentrifuge using an An-60 Ti rotor. Solutions were prepared initially in PBS with peptide concentrations of 120 μM for the heterodimeric coiled coils and 10 and 60 μM for CC-B_N⁴ and spun at speeds in the range of 20 000–50 000 rpm. Data sets were initially fitted to a single, ideal species model using Ultrascan (<http://www.ultrascan.uthscsa.edu/>). The partial specific volume (\bar{v}) for each of the various peptides (Tables S5 and S6) and the solvent density (1.0054 g/mL) were calculated using Sednterp (<http://www.jphilo.mailway.com/download.htm>). Errors were obtained in 95% confidence limits using Monte Carlo analysis.

Disulfide-Exchange Experiments. The four-heptad basic peptides (CC-B₁⁴, CC-B_N⁴) were synthesized as *N*- and *C*-terminally Cys(StBu)-modified variants: ^{N-Cys}CC-B₁⁴, ^{C-Cys}CC-B₁⁴, ^{N-Cys}CC-B_N⁴, and ^{C-Cys}CC-B_N⁴. The corresponding four-heptad acidic peptides (CC-A₁⁴, CC-A_N⁴) were synthesized with the *N*-terminal Cys(StBu) modification only. The mixing experiments for the CC-A₁B₁ and the CC-A_NB_N pairs were carried out under closely similar conditions: mixing 50 μM peptide concentration for each of the basic peptides and 100 μM peptide concentration for the acidic peptides in a PBS redox buffer containing 4 mM oxidized glutathione and 1 mM reduced glutathione. The samples were left to equilibrate for 20 h. The reactions were analyzed by reversed-phase HPLC (100 μL of the reaction mixture) and MALDI-TOF mass spectrometry (I-series, see Figure S2; N-series, see Figure S3).

RESULTS

Peptide Design. Basic design rules for heterodimeric coiled coils include using β -branched amino acids such as isoleucine (Ile, I) at *a* combined with leucine (Leu, L) at *d* to specify dimers.¹⁰ Heterospecificity and the orientation of the α -helices in dimeric coiled coils can then be guided by complementary charged residues at the *e* and *g* positions, e.g., lysine (Lys, K) and glutamic acid (Glu, E).^{11,12,36} Although more-elaborate designs can be made,^{30,31,37} the simplest solution is to make one component with all Lys at *e* and *g* (the basic peptide) and the other with all Glu at these sites (the acidic peptide). Using these guidelines, we designed three peptide pairs with an Ile-Leu core: alanine (Ala, A) as a small, helix-favoring residue at the *b* and *c* positions, and at the *f* positions glutamine (Gln, Q), Lys, and tyrosine (Tyr, Y, basic peptides) or tryptophan (Trp, W, acidic peptides) to increase solubility and to add UV chromophores. In an attempt to access different stabilities, the lengths of the three peptide pairs were varied between three and four heptads (Figure 1A, Table 1). Each peptide was capped with glycine (Gly, G) at the *N*-terminus and a glycine-serine (Ser, S) tag at the *C*-terminus. Peptide pairs are named CC-A₁³B₁³, CC-A₁^{3.5}B₁^{3.5}, and CC-A₁⁴B₁⁴ (for coiled coil; acidic

Table 1. Sequences for the Designed Heterodimeric Coiled Coils

	Sequence and heptad register ^a			
	<i>gabcdef</i>	<i>gabcdef</i>	<i>gabcdef</i>	<i>gabcdef</i>
CC-A _X ³ B _X ³	G	EIAALEK	EXA ³ ALEW	EIAALEQ GZ
	G	KIAALKY	KXA ³ ALKK	KIAALKQ GZ
CC-A _X ^{3.5} B _X ^{3.5}	G	LEQ EIAALEK	EXA ^{3.5} ALEW	EIAALEQ GZ
	G	LKQ KIAALKY	KXA ^{3.5} ALKK	KIAALKQ GZ
CC-A _X ⁴ B _X ⁴	G	GEIAALEQ	EIAALEK	EXA ⁴ ALEW EIAALEQ GZ
	G	GKIAALKQ	KIAALKY	KXA ⁴ ALKK KIAALKQ GZ

^aPeptides were synthesized as C-terminal amides and were acetylated at the N-terminus. I-series, X = I, Z = S; N-series, X = N, Z = G.

or basic; and with the index giving the number of heptad repeats).

Solution-Phase Biophysical Characterization. The designed peptides were synthesized by solid-phase peptide synthesis, and characterized for structure and thermal stability using CD spectroscopy. The CD spectra showed the characteristic minima at 208 and 222 nm, indicating α -helical structures for all three peptide pairs, and 70–85% helicity (Figure 2C, Table S1). Furthermore, thermal-denaturation experiments revealed high thermal stabilities for all three combinations (Figure 2E). However, only CC-A_I³B_I³ gave a true sigmoidal curve (melting temperature T_M = 68 °C) consistent with a cooperatively folded discrete structure. CC-A_I^{3.5}B_I^{3.5} and CC-A_I⁴B_I⁴ remained largely folded up to 90 °C and showed minor transitions at relatively low temperatures (28 and 40 °C). We tested for kinetically trapped peptide assemblies, as the individual peptides for these pairs did form relatively stable homomers (Figure 3A,C) with T_M in the range of 22–67 °C. However, the thermal-unfolding data for the pairs were reproducible, even after controlled heating and cooling. Though the three-heptad sequences did appear to form heterodimeric coiled coils, as the peptides were extended, non-sigmoidal thermal-denaturation curves were obtained indicating that several structural states were accessible.

Next, we performed AUC to determine the oligomeric state of the designed peptide assemblies. We found that CC-A_I³B_I³ was folded as a heterodimeric species, whereas the longer peptide pairs appeared to be mixtures of dimers and higher oligomers (Table S7).

Disulfide-Exchange Experiments. To determine the orientation of the heteroassemblies, we performed disulfide-exchange experiments, concentrating on CC-A_I⁴B_I⁴. Two variants of CC-B_I⁴ modified with cysteine (Cys, C) at the N-terminus and the C-terminus, respectively, and CC-A_I⁴ as an N-terminally modified variant only (Table S9) were synthesized. The sequences were designed to match as closely as possible the unmodified peptides. Analysis of the CC+ database³⁹ revealed that 99.5% of antiparallel coiled-coil peptides have an offset of ~1 residue on average and the N-terminus is shifted towards the coiled-coil center. Based on this, we designed the C-terminally modified peptides with a single Gly linker and the N-terminally modified peptides with a double Gly linker. Furthermore, each peptide and the disulfide-bonded combinations were designed to have unique masses. The Cys residues were protected as *tert*-butyl-cysteiny disulfides to minimize undirected disulfide formation due to air oxidation. Thus, depending on the orientation of the peptides in the assembly, N^{Cys}-CC-A_I⁴ could form a disulfide with either N^{Cys}-CC-B_I⁴

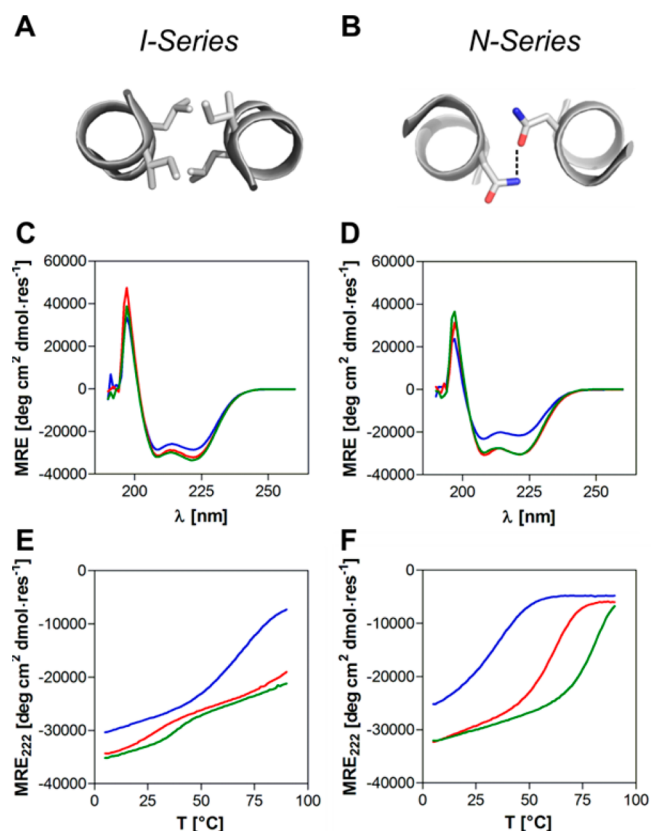


Figure 2. (A,B) One-heptad slices through the X-ray crystal structures, showing the Ile-Leu core of the basis-set dimer CC-Di (A, PDB ID 4DZM)¹⁸ and Asn@ α -containing heptad of the GCN4 leucine zipper (B, PDB ID 2ZTA).³⁸ (C,D) CD spectra (20 °C) of the CC-A_IB_I peptides (C) and CC-A_NB_N peptides (D). (E,F) Thermal-denaturation curves (222 nm) of the CC-A_IB_I peptides (E) and CC-A_NB_N peptides (D). Key: (C,E) CC-A_I³B_I³, blue; CC-A_I^{3.5}B_I^{3.5}, red; CC-A_I⁴B_I⁴, green. (D,F) CC-A_N³B_N³, blue; CC-A_N^{3.5}B_N^{3.5}, red; CC-A_N⁴B_N⁴, green. Conditions: phosphate-buffered saline, 100 μ M total peptide concentration.

(parallel orientation) or C^{Cys}-CC-B_I⁴ (antiparallel orientation). As shown in Figure 4A, mainly the disulfide corresponding to the parallel heterodimer (N^{Cys}-CC-A_I⁴B_I⁴) was detected by HPLC (35% based on area% in HPLC) and MALDI-TOF analysis (Figure S2). However, trace amounts (~6% based on area% in HPLC) of the disulfide-linked antiparallel dimer (N^{Cys}-CC-A_I⁴B_I⁴) were also found.

Increasing the Dimer Specificity. The biophysical studies of the CC-A_IB_I peptides (I-series) revealed the need to revisit our initial design of parallel heterodimeric coiled coils. A similar heterodimeric coiled-coil design with the basic:acidic peptide set up, and the Ile-Leu core is reported to guide a dimeric assembly.^{27,28} However, the longer peptides of our I-series appear to assemble into more-complex ensembles. Furthermore, the stabilities are very high, with even the three-heptad pair having a nanomolar dissociation constant (CC-A_I³B_I³: K_d = 6.64×10^{-9} M, Figure S4A). Clearly, the promiscuity of these sequences is not acceptable, and even the high stability may have disadvantages for broad applicability in synthetic biology. Therefore, to address these issues we added another design feature, namely, incorporating an asparagine (Asn, N) residue at the α position of a central heptad (Figure 1B, Table 1). Pairs of Asn residues are widely found in homo- and heterodimeric Leu zipper domains of transcription factors.^{38,40} The hydro-

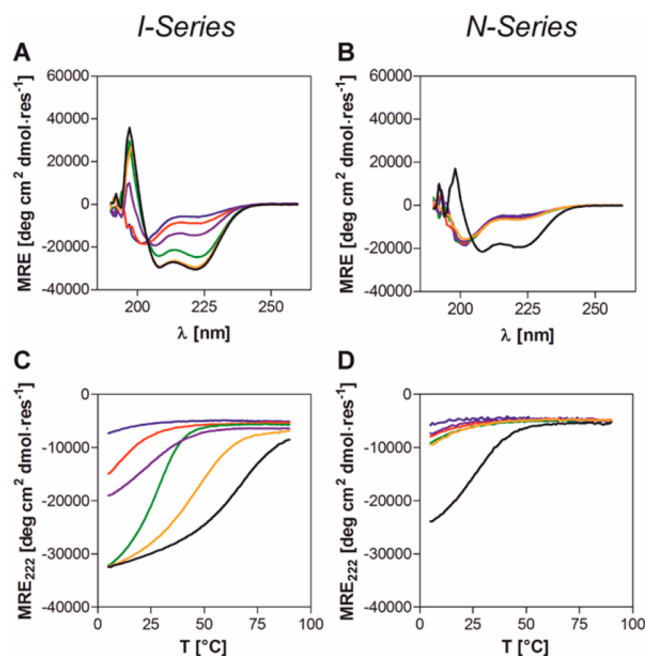


Figure 3. (A,B) CD spectra (20 °C) of the individual peptides of the I-series (A) and the N-series (B). (C,D) Thermal-denaturation curves (222 nm) of the individual peptides of the I-series (C) and N-series (D). Key: (A,C) CC-A_I³, blue; CC-A_I^{3,5}, red; CC-A_I⁴, green; CC-B_I³, purple; CC-B_I^{3,5}, yellow; CC-B_I⁴, black. (B,D) CC-A_N³, blue; CC-A_N^{3,5}, red; CC-A_N⁴, green; CC-B_N³, purple; CC-B_N^{3,5}, yellow; CC-B_N⁴, black. Conditions: phosphate-buffered saline, 100 μM total peptide concentration.

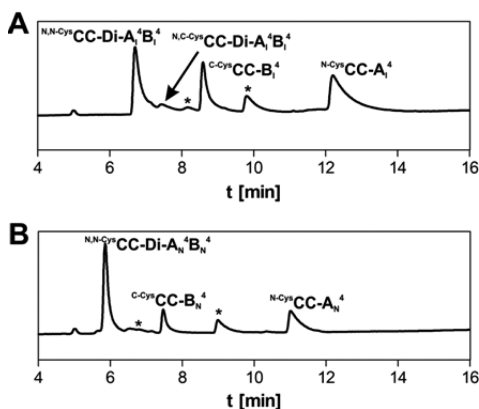


Figure 4. Representative HPLC traces of the disulfide-exchange experiments for CC-A_I⁴B_I⁴ (A) and CC-A_N⁴B_N⁴ (B) conducted with Cys(StBu)-tagged monomer peptides. Conditions: 50 μM N-Cys-CC-B_X⁴, 50 μM C-Cys-CC-B_X⁴, 100 μM N-Cys-CC-A_X⁴ (X = I, N); PBS, 1 mM reduced glutathione, 4 mM oxidized glutathione. Asterisk indicates glutathione-monomer adducts.

philicity of Asn when placed at *a* destabilizes the hydrophobic core, but for reasons that are not entirely understood it also improves the dimer specificity;¹⁸ a common explanation for the second effect is that the Asn residues form a hydrogen bond (Figure 2B). The redesigned peptide pairs were named as CC-A_N³B_N³, CC-A_N^{3,5}B_N^{3,5}, and CC-A_N⁴B_N⁴ (the N-series). To distinguish between I- and N-series, we added a C-terminal Gly instead of a Ser as a mass tag (Table 1).

Investigation of the secondary structure, thermal stability, and oligomeric state revealed that the shortcomings of our initial design were ameliorated by the I-to-N point mutations.

From the CD spectra the three peptide pairs were α -helical (Figure 2D), although helicities were reduced (54–76%, Table S3) compared to the I-series, as expected. The thermal stabilities of the assemblies were also reduced. However, and importantly, clean sigmoidal curves were observed in all three cases, giving T_M values in the range 34–80 °C (Figure 2F, Table S4). Moreover, the individual peptides were mainly unfolded; only CC-B_I⁴ gave a relatively weak homomeric assembly with $T_M = 24$ °C (Figure 3B,D). AUC data for CC-B_I⁴ were fitted to monomer–dimer equilibrium and gave $K_d = 48$ μM (Figure S11), thus fulfilling another of our design objectives of having off–on folding and assembly: “off” for individual peptides and “on” only for the mixtures. Others have shown that off–on folding in heterodimeric coiled-coil design can also be achieved with an all-Leu hydrophobic interface and oppositely charged peptide strands;¹¹ however, the reduced coiled-coil stability was compensated by the five-heptad chain lengths of the individual peptides.

From AUC measurements we found that peptide pairs of the N-series formed the desired heterodimers (Table S8). The orientation of the CC-A_NB_N complexes was shown to be all-parallel by disulfide-exchange experiments with Cys-tagged variants of CC-B_N⁴ and CC-A_N⁴ (Figure 4B). The presence of the antiparallel species was not detected, at least as far as the sensitivity of these experiments allows.

The results of this second round of design and biophysical experiments confirm our goal of achieving a set of robust parallel heterodimeric coiled coils for the N-series. On this basis, these sequences can be renamed simply as CC-Di-AB, indicating that they are coiled-coil, A-B-type heterodimers, and added to the basis set or toolkit of coiled coils and the associated Pcomp database.¹⁸

Mixing Experiments. Next we considered mixing peptides of different lengths (Table 2). Another assumed role for conserved Asn@*a* in natural coiled coils is that they prevent slippage of the helices relative to each other. This is because unpaired Asn residues will cause greater destabilization of the core. For the mixing experiments of the N-series peptides this should carry the consequence of bringing the peptides together in-register, even for combinations of peptides of different

Table 2. Sequences for the Heterodimeric Coiled Coils with Different Lengths of the Individual Peptides

	Sequence and heptad register ^a			
	abcdef	gabcdef	gabcdef	gabcdefg
CC-Di-A _N ³ B _N ^{3,5}	G	EIAALEK	ENAALEW	EIAALEQ GG
	G	LKQ	KIAALKY	KNAALKK KIAALKQ GG
CC-Di-A _N ³ B _N ⁴	G	EIAALEK	ENAALEW	EIAALEQ GG
	G	GEIAALKQ	KIAALKY	KNAALKK KIAALKQ GG
CC-Di-A _N ^{3,5} B _N ³	G	LEQ	EIAALEK	ENAALEW EIAALEQ GG
	G		KIAALKY	KNAALKK KIAALKQ GG
CC-Di-A _N ^{3,5} B _N ⁴	G	LEQ	EIAALEK	ENAALEW EIAALEQ GG
	G	GKIAALKQ	KIAALKY	KNAALKK KIAALKQ GG
CC-Di-A _N ⁴ B _N ³	G	GEIAALEQ	EIAALEK	ENAALEW EIAALEQ GG
	G		KIAALKY	KNAALKK KIAALKQ GG
CC-Di-A _N ⁴ B _N ^{3,5}	G	GEIAALEQ	EIAALEK	ENAALEW EIAALEQ GG
	G	LKQ	KIAALKY	KNAALKK KIAALKQ GG

^aPeptides were synthesized as C-terminal amides and were acetylated at the N-terminus. I-series, X = I, Z = S; N-series, X = N, Z = G.

lengths. This increased complexity opens possibilities for adjusting coiled-coil heterodimer stabilities simply by mixing and matching.

To test these ideas, first we performed CD spectroscopy and CD thermal-denaturation experiments for the six possible mixtures: α -helical structure was found in all cases (Figure 5A); they all exhibited cooperative unfolding with T_M values in the range 42–67 °C (Figure 5B, Table S4); and all were dimeric assemblies as confirmed by AUC experiments (Table S8).

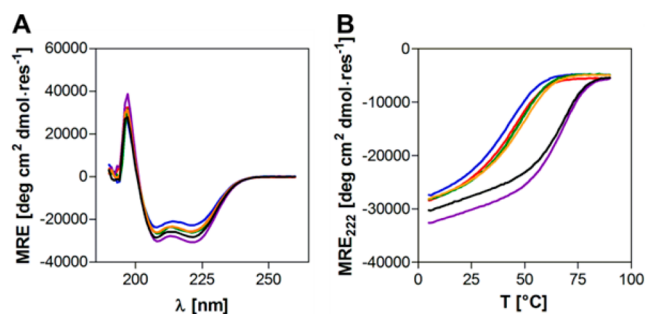


Figure 5. (A) CD spectra of the CC-Di- $A_N B_N$ peptides with different lengths of the individual peptides recorded at 20 °C. (B) Thermal-denaturation curves of the CC-Di- $A_N B_N$ peptides with mixed lengths recorded at 222 nm. Key: CC-Di- $A_N^3 B_N^{3.5}$, blue; CC-Di- $A_N^3 B_N^4$, red; CC-Di- $A_N^{3.5} B_N^3$, green; CC-Di- $A_N^{3.5} B_N^4$, purple; CC-Di- $A_N^4 B_N^3$, yellow; CC-Di- $A_N^4 B_N^{3.5}$, black. Conditions: phosphate-buffered saline, 100 μ M total peptide concentration.

K_d Measurements. To quantify the strengths of the interactions more formally and more usefully for protein engineering and synthetic biology, we determined dissociation constants (K_d). For these, CD–thermal-denaturation experiments were conducted over a range of concentrations.⁴¹ From the different melting temperatures, the concentrations at which the complexes were 50% folded for a given temperature were determined and then related to the K_d value (Figure S4B–J). For the N-series, we found K_d values in the micromolar to subnanomolar range (Table 3). The weakest interaction was observed for CC-Di- $A_N^3 B_N^3$. Increasing the lengths of both peptide chains by one helix turn resulted in a 600-fold improvement in the K_d , presumably due to the additional Leu-Leu interaction. The four-heptad heterodimer gave the most stable complex with K_d in the picomolar range. Interestingly, the extension of only one peptide strand influenced significantly heterodimer stability. This can be partly explained with additional Leu-Leu or Ile-Ile pairings. For instance, there is a 60-fold difference in the K_d values of CC-Di- $A_N^{3.5} B_N^3$ and CC-Di- $A_N^{3.5} B_N^{3.5}$. But even when the hydrophobic core is not extended, increases in the coiled-coil stabilities were observed. We assume that the α -helical peptide overhang stabilizes the N-terminus from fraying due to an increase in the number of nucleation sites⁴² and/or the steric bulk. Furthermore, N-capping seem to influence the stability of the peptide assemblies

because heterodimeric coiled coils with a Glu-rich overhang are slightly more stable than those with a Lys-rich overhang.⁴³

DISCUSSION AND CONCLUSIONS

In summary, we have shown that for the *de novo* design of parallel heterodimeric coiled coils, entirely Ile-Leu cores do not reliably guide parallel dimeric assemblies. However, this can be corrected by including Asn residues at the *a* site of a central heptad of the individual peptides. Based on this, we present a new and reliable set of parallel heterodimeric α -helical coiled coils with variable lengths from three to four heptads. Due to the presence of the Asn residues, assemblies of peptides of different lengths have also been made accessible, and in this way the stabilities of these complexes have been tuned. Therefore, we proffer that this set of peptides, dubbed CC-Di-AB, is a useful addition to the toolkit of rationally designed coiled-coil peptides¹⁸ and that they will find applications in protein engineering and synthetic biology where protein–protein interactions of defined stabilities are required.

The new designs are straightforward and predictable in terms of folding and complex stability, but they also leave space for modification, notably at the solvent-exposed and non-oligomer-state-specifying residues' *b*, *c*, and *f* positions and also at their termini,^{8,44,45} though we have not demonstrated this directly in this report. In contrast to other designs, the emphasis was not on obtaining very stable coiled-coil interactions but on achieving specificity and tuning stability. Our view is that very stable complexes may be restricted in their applicability in protein engineering and synthetic biology. In fact, a broad range of stabilities is desirable for applications other than straightforward oligomerization and association, for instance, in more-complex association–dissociation processes involving complex dynamics and subunit exchange. The set of parallel heterodimeric coiled coils presented here may help pave the way to such applications.

ASSOCIATED CONTENT

Supporting Information

Additional figures and tables, characterization data, and the K_d measurement procedure. This material is available free of charge via the Internet at <http://pubs.acs.org>. The CC-Di- $A_N B_N$ peptides have been uploaded to the PComp database, which is freely accessible (<http://coiledcoils.chm.bris.ac.uk/pcomp/>).

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Notes

The authors declare no competing financial interest.

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Table 3. K_d Values (M) at 20 °C of the CC- $A_N B_N$ Peptides

	CC- A_N^3	CC- $A_N^{3.5}$	CC- A_N^4
CC- B_N^3	$(3.10 \pm 0.58) \times 10^{-6}$	$(2.79 \pm 1.42) \times 10^{-7}$	$(7.47 \pm 3.30) \times 10^{-8}$
CC- $B_N^{3.5}$	$(6.40 \pm 2.77) \times 10^{-7}$	$(5.15 \pm 2.05) \times 10^{-9}$	$(2.08 \pm 0.73) \times 10^{-10}$
CC- B_N^4	$(3.87 \pm 1.45) \times 10^{-7}$	$(4.74 \pm 4.49) \times 10^{-10}$	$<1.0 \times 10^{-10}$

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