An Effective Strategy for Stabilizing Minimal Coiled Coil Mimetics

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S *Supporting Information*

ABSTRACT: Coiled coils are a major motif in proteins and orchestrate multimerization of various complexes important for biological processes. Inhibition of coiled coil-mediated interactions has significant biomedical potential. However, general approaches that afford short peptides with defined coiled coil conformation remain elusive. We evaluated several strategies to stabilize minimal helical bundles, with the dimer motif as the initial focus. A stable dimeric scaffold was realized in a synthetic sequence by replacing an interhelical ionic bond with a covalent bond. Application of this strategy to a more challenging native protein−protein interaction (PPI) suggested that an additional constraint, a disulfide bond at the internal a/d' position along with a linker at the e/e' position, is required for enhanced conformational stability. We anticipate the coiled coil stabilization methodology described herein to yield new classes of modulators for PPIs.

Mimicry of critical secondary structure motifs that mediate protein−protein interactions (PPIs) offers a promising approach for the discovery of new classes of therapeutics.[1] Several inhibitors of helical protein interfaces have been described, owing to the high occurrence of helices at PPI interfaces[2] and the development of synthetic approaches that enabled mimicry of this secondary structure.[3] Examination of PPI interfaces suggests that many complexes often utilize contacts from multiple helices, and that these complexes will potentially require inhibitors that are capable of interactions beyond mimicry of a single helix.[4] These multi-helix interfaces commonly comprise individual helices from the α-helical coiled coil motif.[5] An example of such an interface is shown in Figure 1, where a protein partner presents critical residues for biomolecular recognition from helices that are part of two-strand coiled coil assemblies. We have undertaken a comprehensive
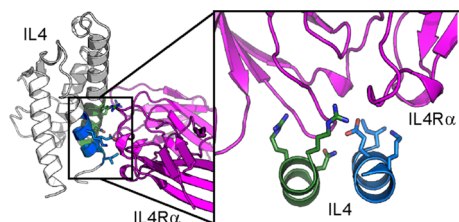

Figure 1. An example of a protein complex that utilizes residues from both helices of a dimeric coiled coil domain to target the partner proteins. Model depicts complex between IL-4 and IL-4 receptor α-chain (PDB code 1IAR).

analysis of high-resolution structures in the Protein Data Bank to identify all PPIs mediated by helix dimers.[4]

Formation of coiled coil assemblies is implicated in many biological processes. Canonical coiled coils are stabilized by a series of hydrophobic knobs-into-holes[5a] packing interactions along with inter- and intrastrand electrostatic contacts.[5b−d] Several helical peptides and peptidomimetic inhibitors that target coiled coil domain assembly in biological processes such as viral fusion have been described.[6] These inhibitors function by inhibiting formation of coiled coil contacts. This strategy may also be applicable to complexes between globular proteins and pre-formed coiled coils, such as the one depicted in Figure 1. An alternative strategy could be to utilize coiled coil mimics or stable helix dimers that display the desired functionality to interact with the globular protein partner.

Our survey of the structural data reveals that typical helical dimers in PPIs span 12−18 residues per helix,[4a] which is consistent with the average length of helices at protein interfaces.[2] A suitable dimeric helix scaffold would thus be capable of spanning this length. The stability of coiled coils, however, is directly proportional to the number of heptad repeats and the correct pairing of the hydrophobic and ionic residues. Coiled coils consisting of less than three heptads are generally not stable.[7] Although highly engineered short coiled coils have been described,[5d,7b,8] these approaches may not be suitable for inhibitor design as at least one face of the dimer is needed to display appropriate functionality to engage the target. We envisioned a synthetic approach that could be applied to stabilize a range of helical dimers in a *sequence-independent* manner.

Coiled coils consist of heptad repeats with critical hydrophobic contacts at the a and d positions and ionic residues at the e and g positions. We hypothesized that a helix capable of a minimum of three a/d hydrophobic contacts (or 1.5 heptads) provides a reasonable starting point for development of minimal coiled coil mimics. We postulated that strategies that stabilize such short helix dimers would also be applicable for longer chains since coiled coil stability increases with number of contacts.[7a] Short, helical dimers can project side chains for biomolecular recognition only if individual helices are packed against each other.[5a] We envisioned four different approaches for the *de novo* design of minimal coiled coil mimics for the stabilization of a model sequence (Figure 2). The model sequence incorporates favorable hydrophobic residues at a/d positions as well as judiciously placed inter- and intrastrand ionic interactions to enhance both the helix and the dimer stability. We created a hydrophobic interface following the recently described design rules for vertical triads.[9] Gellman and Woolfson et al. suggest that
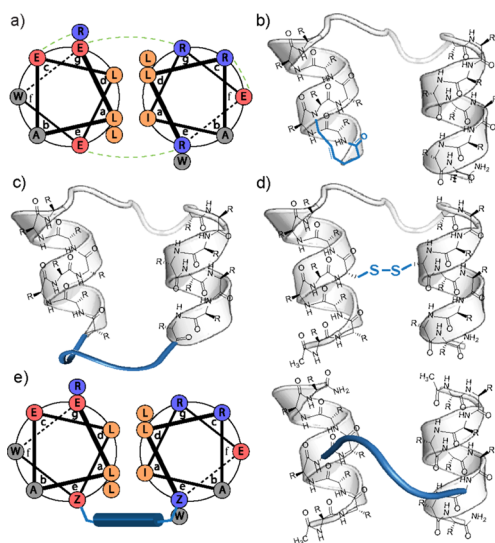
Figure 2. Strategies to template coiled coil formation of designed peptides. (a) A potential antiparallel coiled coil assembly between peptides **A** and **B**. (b) Use of H-bond surrogate helices to stabilize helical dimers, (c) macrocyclization of peptides, (d) use of interhelical disulfide bridges in place of hydrophobic contacts to aid assembly, and (e) placement of covalent bonds in place of interstrand ionic interactions.

placement of Leu-Ile-Leu residues at a-a'-a positions contributes significantly to helical dimer stability because of optimal packing interactions.[9] We positioned intra- and interhelical salt bridges at appropriate positions to enhance stability of coiled coil assemblies.[5c,d,10] These design considerations led to peptides **A** (Ac-ELAELEWRL-NH$_2$) and **B** (Ac-LWERIARLR-NH$_2$). Potential inter- and intrastrand interactions between **A** and **B** in the context of an antiparallel coiled coil are depicted in Figure 2a.

Seminal work investigating the stabilities of minimal, *de novo*-designed coiled coils suggests that our designed peptides (**A** and **B**) would not spontaneously assemble in aqueous solution,[7] because (a) short peptides do not adopt stable helical conformations[11] and (b) short helices do not create enough contacts to favor dimer assembly. Circular dichroism (CD) spectroscopy was used to assess the conformational stability of peptides. CD provides a distinct signature for α-helices with a maximum near 190 nm and minima at 208 and 222 nm.[12] The relative helicity of peptides is typically estimated by the mean residue ellipticity at 222 nm,[12,13] although these estimates are often not accurate for short helices.[14] The ratio of the 222/208 nm bands offers an additional gauge of α-helicity. The origin and effect of peptide sequence on this ratio remain ill-defined,[12] but a ratio of ≥ 1 is expected of stable α-helices.[15] CD results displayed nonhelical signatures for each individual peptide (**A** and **B**) and their equimolar mixture at 20 μM concentration (Supporting Information (SI), Figure S1).

We evaluated the potential of four synthetic strategies to create conformationally defined coiled coil mimics (Figure 2). The minimal mimetic designs build on the following key hypotheses: (a) Stabilization of individual helices will enhance stability of the dimeric assembly, and coil formation in an attached peptide can be nucleated with a preformed helix.[16] (b) Macrocyclization of the dimeric scaffold would aid interpeptide contacts and helix formation.[16a] (c) Noncovalent interhelical contacts can be strengthened by substitution with covalent bonds.[17] Our studies reveal that replacement of an interhelical ionic bond with a covalent bond provides a general and versatile approach for

stabilization of short helix dimers. We extensively characterized the constrained, antiparallel coiled coil mimics by CD and 2D NMR spectroscopies and then applied the design to the modulation of a PPI involved in leukemogenesis, where complex formation depends on coiled coil assembly.

We began by determining if a preformed helix could nucleate helical conformation in an attached peptide. We utilized the H-bond surrogate (HBS)[17a] strategy to stabilize the helical conformation in peptide **A**, and installed a GGSSGG linker[9] between HBS-**A** helix and peptide **B** to access **AB-1** as a potential antiparallel helix-loop-helix motif. However, CD studies indicated a weakly helical signature in **AB-1** reminiscent of a single short helix stabilized by the HBS approach (Figure S1).

We next tested whether macrocycliclization of peptides A and B with two GGSSGG loops (**AB-2**: cyclo(GGSSGGELAELE-WRLGGSNGGLAERIARLR) could induce helical dimer association in both sequences (Figure 2b). This scaffold would potentially limit fraying at all four peptide termini while promoting interstrand hydrophobic interactions. Again, CD spectroscopy revealed minimal helicity, suggesting that macro-cyclization did not lead to a significant conformational stability relative to the HBS strategy (Figure S1). Subsequently, we sought to determine if an interhelical disulfide linkage in place of hydrophobic pairing would lead to a stable dimer. Hodges et al. demonstrated that mutation of hydrophobic residues to create disulfide bridges increases coiled coil stability while preserving coiled coil structure.[17b] Their seminal work serves as the basis of our disulfide design. We synthesized a bis-cysteine peptide (**AB-3**: ECAELEWRLGGSSGGLAERIARCR) on resin followed by disulfide formation and characterized its helical content by CD. Analysis revealed that this approach also did not provide significant helical stabilization in short sequences.

Salt-bridge networks contribute significantly to coiled coil chain alignment as well as to general helix stability;[5d,18] although there is debate,[19] individual salt-bridges are thought to stabilize helices and coiled coils by ≤ 0.5 kcal/mol.[20] We envisioned that replacement of a weak interhelical ionic bond at g/g' or e/e' positions with a covalent bond would offer an attractive option for stabilizing helical dimers. Although many covalent constraints can be envisioned, in this preliminary investigation, we designed bis-triazole linkers formed via copper-catalyzed azide−alkyne cycloaddition reaction to constrain peptides **A** and **B** (Figure 3).[16a,21] Bis-triazole bridges of varying lengths resulting from azidoalanine, azidohomoalanine, and azidolysine residues were incorporated at positions e/e' to obtain dimers **AB-4**, **AB-5**, and **AB-6**, respectively (Figure 3a). The azido side chains were reacted with propargyl ether to obtain the bis-triazole linkers. Solid-phase synthesis of **AB-4**−**AB-6** is described in Figure S2. CD analysis reveals that replacement of an ionic bond with a covalent linkage has a dramatic effect on the conformational stability in a linker-length-dependent manner (Figure 3b). Based on the intensity of the 222 nm minimum and 222/208 nm ratio, we find **AB-4** and **AB-5** constructs derived from azidolysine and azidohomoalanine, respectively, to be significantly more helical than **AB-6**.

The conformational stability of the cross-linked helix dimer (CHD) **AB-4** (Figure 3c) was further assessed using a combination of 1D NMR, total correlation spectroscopy (TOCSY), and nuclear Overhauser effect spectroscopy (NOESY) in 10% d_3-CH$_3$CN in H$_2$O with 0.1% TFA (pH 5). We found addition of 10% acetonitrile to be necessary to limit aggregation of the peptide at the 0.5 mM concentration needed for NMR. The NOESY spectrum revealed NOE crosspeaks
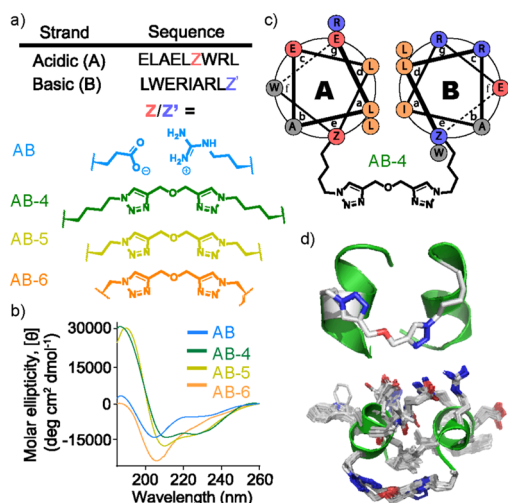
Figure 3. (a) Design of cross-linked helix dimers by replacement of an interstrand ionic contact with bis-triazole linkers. Bis-triazole linkers of varying lengths resulting from azidoalanine, azidohomoalanine, and azidolysine residues were incorporated at coiled coil positions e/e' to obtain dimers **AB-4**, **AB-5**, and **AB-6**, respectively. (b) CD spectra of **AB-4**−**AB-6** in 50 mM aqueous KF, pH 7.4. (c) Helical wheel diagram of **AB-4**. (d) NMR-derived structure of **AB-4**. The lowest conformer (top) and ensemble of 20 lowest conformers (bottom).

indicative of a helical tertiary structure, showing sequential dNN $(i,i+1)$ and several medium range NOEs $(d\alpha N\ (i,i+3))$ suggestive of stable helices (SI, Figure S3). Additionally, the backbone dihedral angles (Φ) calculated from $^3J_{NHCH\alpha}$ coupling constants fall in the range expected for canonical α-helices. A structural model of **AB-4** was calculated using 65 NOESY crosspeaks and 18 Φ constraints (Figure 3d).

To establish that the CHD strategy can be translated from a designed sequence to a native protein coiled coil, we developed mimics of Nervy homology two (NHR2) domain of the AML1-ETO-containing transcription factor complex which interacts with NHR2-binding (N2B) motif of E-proteins.[22] This complex is critical for leukemogenesis and features a dimeric, antiparallel coiled coil from NHR2 at the interface to engage N2B (Figure 4a). Computational alanine scanning (SI, Table S1) and experimental mutagenesis data[22] reveal residues E501, H504, L508, V522, and S525 as keys for binding.

To investigate the potential of a bis-triazole bridge to induce stable, dimeric helical conformation in an NHR2 sequence, we inserted azidolysine residues at the e/e' position of the native sequence to obtain **CHD-NHR2-1**: ^{g}EWKHLZHLLN^b/^{c}ELW-RSIRVLZ$^{e'}$ (Figure 4b). CD spectroscopy showed this construct to be largely nonhelical (Figure 4c). We attribute this result to the missing stabilizing contribution from the hydrophobic vertical triad, since the native sequence contains potentially disruptive large tryptophan and polar threonine within the interior of its hydrophobic core.[23] The native sequence also contains two positively charged residues near the amino terminus, which likely reduce the helical stability. We redesigned **CHD-NHR2-1** to include the optimal hydrophobic residues from **AB-4** and intrahelical salt-bridges at the i and $i+3$ positions while preserving the native residues that interact with N2B to obtain **CHD-NHR2-2**: ^{g}ELWHLZELLR^b/$^{c'}$ELWRSIRVLZ$^{e'}$. The redesigned sequence is significantly more helical than the parent, as ascertained by the intensity of the 222 nm minimum and the ratio of the 222/208 nm bands (Figure 4c), but the
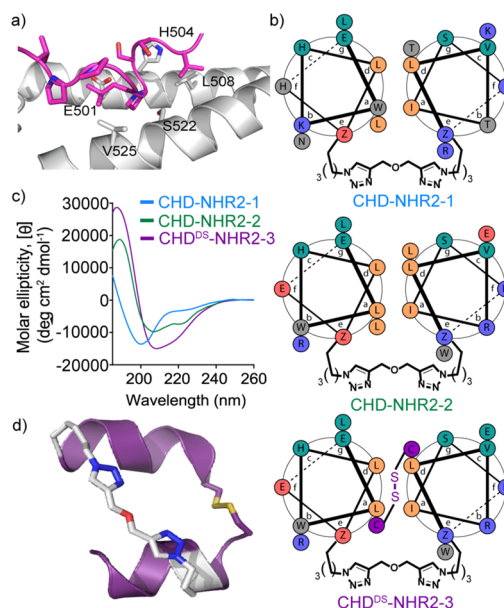

Figure 4. (a) Model depicting binding of NHR2 (gray) to N2B (magenta) with critical residues labeled. PDB code: 4JOL (b) Helical wheel diagrams depicting sequences for the native (**CHD-NHR2-1**) and the redesigned (**CHD-NHR2-2**) sequences. Z = azidolysine-derived bis-triazole linker. (c) CD spectra of **CHD-NHR2-1**, **CHD-NHR2-2**, and **CHD^{DS}-NHR2-3** in 50 mM aqueous KF, pH 7.4. (d) Computational model and helical wheel diagram of **CHD^{DS}-NHR2-3**.

overall helical stability of this native sequence remained low (θ_{222} < 10 000) as compared to the designed sequence **AB-4** (θ_{222} = 14 000). This result prompted us to reevaluate our stabilization approach to determine if further constraints can be placed to stabilize the dimer in the context of difficult biological sequences. Placement of more than one linker at the g/g' position is not desirable as it would influence the binding surface. Although the internal disulfide bridge did not offer significant stability in the context of a flexible tether (**AB-3**), we sought to determine the effect of interhelical disulfide bonds in enhancing stability of triazole cross-linked dimer **CHD-NHR2-2**.

Disulfide bridges may be placed at different a/d positions within **CHD-NHR2-2** such that they are located adjacent to the triazole link at e/e' positions or farther away (Figure 4d and SI, Figure S4). We conjectured that placement of the disulfide bond farthest away from the triazole bridge would have the highest impact on helix stability as the designed salt bridge surrogate may not be an optimal helix nucleator.[17a] Our results support this hypothesis. **CHD^{DS}-NHR2-3** in which the disulfide is located distal from the triazole bridge is significantly more helical, according to CD spectroscopy, than **CHD^{DS}-NHR2-4** and **CHD^{DS}-NHR2-5**, where the disulfide bonds are placed near the triazole linker (Figure S4). **CHD^{DS}-NHR2-3** is also significantly more helical than **CHD-NHR2-2**, with the overall CD signature similar in intensity to that of **AB-4** (Figure 4c).

Next, we determined the binding affinities of the designed NHR2 mimetics to correlate their molecular recognition attributes with the conformational stability. We utilized a previously described fluorescence polarization assay with a fluorescein-labeled N2B peptide to evaluate binding of the cross-linked dimers as compared to the native NHR2 coiled coil (NHR2$_{482-551}$).[22] The native NHR2 domain binds to the N2B peptide in agreement with published results (K_d = 356 ± 90 μM), while **CHD-NHR2-1**, **CHD-NHR2-2**, and **CHD^{DS}-NHR2-3**

target N2B with K_d values of >10 000, 236 ± 90, and 53 ± 20 μM respectively, highlighting the influence of conformational stability on molecular recognition. As expected, the doubly cross-linked dimer, with higher conformational stability, binds to the target with the highest affinity. The 5-fold enhanced affinity of the much shorter **CHD^{DS}-NHR2-3** (20 residues) mimetic versus the native NHR2 coiled coil (138 residues) is notable and supports our design principles.

Lastly, we investigated if contacts from both helices are required for binding to N2B peptide, i.e., if the dimeric construct is necessary for interacting with the target peptide. Residues S522 and V525 on one helix strand and E501 and L508 on the opposite strand are suggested to be critical for binding. **CHD-NHR2-6** (S522A/V525A) and **CHD-NHR2-7** (E501A/L508A) were designed as controls for **CHD-NHR2-2** and contain alanine mutations on one strand per dimer while retaining a **CHD-NHR2-2** sequence on the other strand (Table 1). Both control

Table 1. Sequences and Binding Affinities of the Native NHR2 Coiled Coil and the Cross-Linked Helix Dimer (CHD) Mimics

compound	sequence[a]	K_d (μM)[b]
NHR2	GST-NHR2(482−551)	356 ± 90
CHD-NHR2-1	EWKHLZHLLN/KTRRSLTVLZ	>10 000
CHD-NHR2-2	ELWHLZELLR/ELWRSIRVLZ	236 ± 90[c]
CHD^{DS}-NHR2-3	ELWHLZELCR/ECWRSIRVLZ	53 ± 20
CHD-NHR2-6	<u>A</u>LWHLZE<u>A</u>LR/ELWRSIRVLZ	>3000
CHD-NHR2-7	ELWHLZELLR/ELWR<u>A</u>IR<u>A</u>LZ	>3000

[a]Z = azidolysine-derived bis-triazole linker; alanine mutations are underlined. [b]Binding affinity calculated using a fluorescence polarization assay with fluorescein-labeled N2B peptide (SI, Figure S12). [c]**CHD-NHR2-2** is not fully soluble at >1 mM concentrations, leading to a noisy upper bound and nonoptimal curve fit.

constructs bound N2B with diminished affinity (K_d > 3000 μM), supporting the requirement of critical residues on each helical strand and our hypothesis that a dimer is needed to engage such PPIs.

In summary, we have investigated various stabilization strategies to design minimal mimics of helical tertiary structures. Our studies reveal that judicious replacement of interhelical ionic contacts with covalent linkages and substitution of internal hydrophobic interactions with disulfide bonds afford stable dimeric helical conformations in difficult biological sequences. We applied the design principles to the stabilization of short sequences from a biological assembly to evaluate the potential of the minimal mimetics to reproduce native binding interactions of much longer protein coiled coils. Given the ubiquity of helical PPIs,[4] we anticipate that the helix tertiary structure mimics will prove to be useful as inhibitors.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b05525.

Synthesis, characterization, CD and 2D NMR spectroscopy data, and description of the binding studies (PDF)

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Notes
The authors declare no competing financial interest.

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