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Letters

Folding-Assisted Peptide Disulfide Formation and Dimerization

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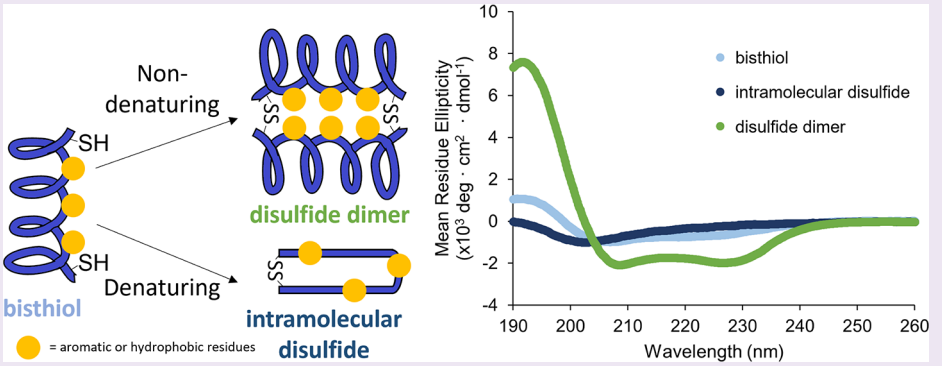

ABSTRACT: Disulfide bonds form covalent bonds between distal regions of peptides and proteins to dramatically impact their folding, stability, and oligomerization. Given the prevalence of disulfide bonds in many natural products, considerable effort has been invested in site-selective disulfide bond formation approaches to control the folding of chemically synthesized peptides and proteins. Here, we show that the careful choice of thiol oxidation conditions can lead to monomeric or dimeric species from fully deprotected linear bisthiol peptides. Starting from a p53-derived peptide, we found that oxidation under aqueous (nondenaturing) conditions produces antiparallel dimers with enhanced α-helical character, while oxidation under denaturing conditions promotes formation of a nonhelical intramolecular disulfide species. Examination across peptide variants suggests that intramolecular disulfide formation is robust across diverse peptide sequences, while dimerization is sensitive to both the α-helical folding of the linear peptide and aromatic residues at the dimerization interface. All disulfide species are more resistant to protease degradation than the linear peptide but are easily reduced to restore the initial bisthiol peptide. Both disulfide formation approaches are compatible with α-helix-stabilizing cross-linkers. These results provide an approach for using disulfide bonds to control peptide folding and oligomerization to better understand how folding influences interactions with diverse molecular targets.

INTRODUCTION

Disulfide bonds are abundant in both natural and designed peptides and proteins. In addition to their role in the structure of large proteins (e.g., antibodies), disulfide bonds play a significant role in many peptide hormones and natural products, including several FDA-approved drugs (e.g., insulin, oxytocin, linaclotide).[1−3] Beyond known drugs, disulfide bonds also play an integral role in several emerging peptide drug scaffolds, where they stabilize specific peptide conformations and improve resistance to protease degradation.[4,5]

The prevalence of disulfide bonds in natural and designed peptides has prompted a wide array of synthetic methods for disulfide bond formation.[6−9] Typically, the method of choice is related to the peptide context, i.e., the number of disulfide bonds and other functional groups present. In peptides with a single disulfide bond, the optimal oxidation method is often determined by the presence or absence of oxidation-sensitive amino acids, particularly methionine, tyrosine, and tryptophan.[10,11] In peptides with multiple disulfide bonds, regioselective disulfide formation is essential and is most often accomplished using stepwise thiol deprotection followed by oxidation.[12,13] Regioselective disulfide bond formation approaches regularly employ a combination of solid-phase or solution-phase techniques. More recently, Brik et al. have described a one-pot method for regioselective formation of up to three disulfide bonds that improves both the speed and yield of peptides containing two to three disulfide bonds.[14,15]

For disulfide bond formation and other macrocyclization approaches, there is inherent competition between the formation of intramolecular and intermolecular species. In most cases, dilute conditions in solution or "pseudo-diluted" conditions (e.g., on low-loading resin) are optimal and sufficient to favor the intramolecular disulfide-bonded

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species.[6,16] Formation of specific intermolecular disulfide-bonded species, especially dimers, is functionally important in several natural peptides (e.g., insulin, fibrinogen)[17,18] and has been reported in certain designed peptides.[19−21]

Here, we find that the balance of intramolecular versus intermolecular disulfide formation can be controlled by tuning the oxidation reaction conditions, folding propensity of the linear peptide, and amino acid sequence. Starting from a p53-derived 14-mer peptide with N- and C-terminal cysteines, reactions with aqueous dimethyl sulfoxide (DMSO) as both cosolvent and oxidant led to exclusive formation of antiparallel peptide dimers with enhanced α-helicity compared to the bisthiol monomer. In contrast, the exclusive formation of intramolecular disulfide bonds was achieved rapidly using disulfiram (DSF) as the oxidant under denaturing conditions and resulted in disruption of α-helical folding. As predicted, disulfide species are more resistant to proteolysis than linear precursors but can be easily reduced to regenerate the monomeric bisthiol species. While intramolecular disulfide formation under denaturing conditions was found to be independent of peptide sequence, dimerization appears to depend on both partial α-helical folding of the linear peptide and alignment and interaction of aromatic residues in helical register with the C-terminal cysteine. Terminal cysteine placement facilitates introduction of additional covalent cross-links to stabilize the initial α-helical structure of the linear bisthiol peptide. Thus, these findings enable controlled bisthiol peptide folding and oligomerization for detailed inquiry into how peptide folding influences interactions with various cellular components, including target proteins and membranes. This strategy may also be useful in the study of aggregation diseases involving helix-to-sheet structural transitions.

RESULTS AND DISCUSSION

Our initial peptide **1**, CTFANLWRLLAQNC, is derived from SAH-p53-8 (QSQQTFX^{1}NLWRLLX^{2}QN), a p53-derived hydrocarbon-stapled peptide with both high α-helicity and high affinity for Mdm2 (X^1 = *R*-2-(7′-octenyl)alanine, X^2 = *S*-2-(4′-pentenyl)alanine).[22,23] In peptide **1**, both olefin-bearing amino acids X^1 and X^2 are replaced with alanine. Removal of the N-terminal QSQQ facilitated the introduction of cysteines at both termini such that intramolecular disulfide formation allows the peptide to fold exactly in half without N- or C-terminal extensions.

Unexpectedly, oxidation of **1** at 0.6−0.7 mM using 20% (v/v) DMSO in a pH 6 aqueous buffer for 24 h failed to produce the target intramolecular disulfide-bonded species. Instead, peptide dimers joined by two disulfide bonds were the sole product (**1-D**, Figures 1 and S1, Table 1). This result was unexpected because disulfide formation in peptides with similar sequences and/or spacing between cysteine residues leads to either intramolecular disulfide formation or requires 2,2,2-trifluoroethanol (TFE) as a helix-inducing solvent to facilitate dimerization.[16,20] Also, the *i*-to-*i*+13 spacing in **1** is one residue short of two full heptads that might be expected to favor helix dimerization with both cysteines on the same helical face.[24]

This initial result prompted a broader survey of disulfide bond-forming approaches from the literature. Attempts to form the desired intramolecular disulfide bond during solid-phase synthesis, including intramolecular disulfide exchange[25] or oxidation using N-chlorosuccinimide,[26] failed to produce well-defined products even under "pseudo-dilution" on low-loading resin. Interestingly, solution-phase reactions using disulfiram (DSF) as the oxidant for 10 min at 37 °C under denaturing conditions (i.e., with concentrated guanidinium chloride, GdCl)[15] was found to yield exclusively the desired intramolecular disulfide species (**1-IM**, Figures 1 and S1, Table 1).

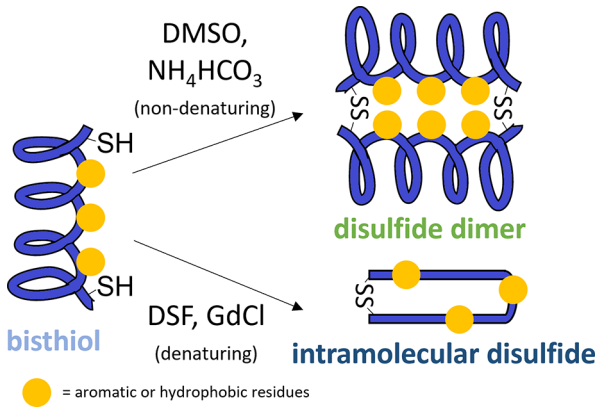

Figure 1. Synthetic control of intramolecular vs intermolecular disulfide formation. Oxidation under nondenaturing conditions yields disulfide-linked dimers while oxidation under denaturing conditions yields monomers with an intramolecular disulfide bond.

Table 1. Products Formed under Different Disulfide Bond-Forming Reaction Conditions

	percent yield[a]		
reaction conditions	**1**[b]	**1-IM**[c]	**1-D**[d]
nondenaturing (N)[e]	1	0	97 ± 3
denaturing (D)[f]	8 ± 4	86 ± 3	0

[a]Yields (± standard deviations) calculated from integration of HPLC chromatograms for at least two replicates each. [b]Initial bisthiol peptide. [c]Intramolecular disulfide. [d]Disulfide-linked dimer. [e]0.66 mM peptide, 20% DMSO in 5.5 mM NH_4HCO_3, pH 6.0, 25 °C, 24 h. [f]0.125 mM peptide, 10 equiv disulfiram (DSF), 5.0 M guanidinium chloride, pH 7.2, 37 °C, 10 min.[15]

A more detailed investigation of factors influencing intramolecular versus intermolecular disulfide formation indicated that nondenaturing (aqueous) conditions favor dimerization to **1-D** while denaturing conditions favor intramolecular disulfide formation to **1-IM** (Table S1). In aqueous DMSO with peptide **1**, with concentrations ranging from 0.1 to 0.66 mM, reactions yielded predominantly dimer **1-D**. Reactions with DSF in 5 M GdCl yielded predominantly intramolecular disulfide **1-IM** as the major product over the same concentration range. As expected, dimerization generally increased with an increase in peptide concentration regardless of reaction conditions, but dilution to 0.10−0.15 mM largely mitigated dimerization under denaturing conditions with DSF.

Switching the oxidants confirmed the dominant role of solution conditions (nondenaturing vs denaturing) in promoting intramolecular disulfide formation or dimerization (Table S1). Using DSF as the oxidant under nondenaturing conditions with the same 24 h reaction time at RT also yielded dimer **1-D** as the major product but with reduced yield (55−65%). Masses consistent with single- and double-diethyldithiocarbamate (DEDTC) adducts (+147 and +294 Da, respectively) were also observed, suggesting that DSF reacted with the peptide to form intermediate mixed peptide-DEDTC disulfides[14] but did not always undergo efficient disulfide exchange

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under aqueous conditions (Figure S2). Reactions with DMSO in concentrated guanidinium chloride at 37 °C did not yield significant amounts of any product (less than 6%) after 10 min as observed with DSF. Extending the reaction time to 60 min produced a mixture of **1** and **1-IM**, but the yield was much lower than the reactions with DSF as an oxidant. Thus, the choice of solution conditions (nondenaturing vs denaturing) was more important to control the final product than the choice of oxidant, but the original oxidant/solution combinations were most efficient.

Structural studies on the bisthiol **1**, intramolecular disulfide **1-IM**, and dimer **1-D** suggest that partial α-helical folding of the bisthiol peptide influenced the propensity for dimerization under nondenaturing conditions. Circular dichroism (CD) studies revealed that bisthiol peptide **1** is weakly α-helical in aqueous buffer based on a weak minimum at 222 nm (Figure 2A). Peptide **1** is ~25% helical based on the ratio of the mean residue ellipticity at 222 nm ($[\theta]_{222}$) and the theoretical maximum α-helicity for a 14-mer peptide.[27,28] The intramolecular disulfide **1-IM** lacks a defined minimum at 222 nm, so it is less helical than **1** (no more than 10−15%). The spectrum for the dimer **1-D** is consistent with increased α-helical folding (~65%) based on the increased intensity at 222 nm; however, precise estimation of helix content from $[\theta]_{222}$ is confounded by a red-shifted minimum at 228 nm, which is consistent with tryptophan and/or disulfide contributions to the CD spectrum.[29−32]

Next, we determined that the peptides in dimer **1-D** have an antiparallel orientation. Trypsin digestion of **1-D** revealed a single digestion product with a mass of approximately 1710 Da, consistent with an antiparallel dimer (Figure 2B,C). The absence of masses of 1318 and 2103 Da further rules out the possibility of a mixture containing parallel dimers. Using orthogonal Trt/Acm protection of cysteine residues, we directly synthesized the parallel dimer **1-D′**, which exhibits a shorter HPLC retention time and slightly reduced α-helicity (~55−60%) compared to that of **1-D** (Figure S3). We speculate that, similar to previous studies with p53-derived peptides,[20] the antiparallel orientation is favored by a combination of enhanced folding, stronger hydrophobic interactions, and/or attraction between helical macrodipoles in the antiparallel orientation.

We also evaluated both protease and disulfide stabilities for **1**, **1-IM**, and **1-D** and found that the disulfide-linked peptides were less susceptible to proteolysis but were easily reduced to the original bisthiol peptide (Figure S4). As a benchmark, the half-life of **1** was approximately 40 s when it was treated with proteinase K, as anticipated for a linear peptide. The half-life of **1-D** was roughly 100 times greater than that of **1** ($t_{1/2}$ = 62 min). Interestingly, **1-IM** proved the most resistant to proteolysis, requiring a 10-fold higher concentration of Proteinase K to observe degradation on the same time scale as the dimer ($t_{1/2}$ = 41 min at 10× proteinase K concentration). As intended, both disulfide species are fully reduced with 10 equiv of tris(2-carboxyethyl)phosphine (TCEP) in 15 min. CD studies show that the α-helical character of **1** is restored when the intramolecular disulfide **1-IM** is reduced for 1 h with TCEP (Figure S5).

To better understand how peptide folding influences intramolecular disulfide vs dimer formation, we initially evaluated two closely related peptide analogs of **1**. Peptide **2**, CTFBNLWRLLBQNC, introduces two α-aminoisobutyric acid (Aib, B) residues in place of the two alanines. Aib

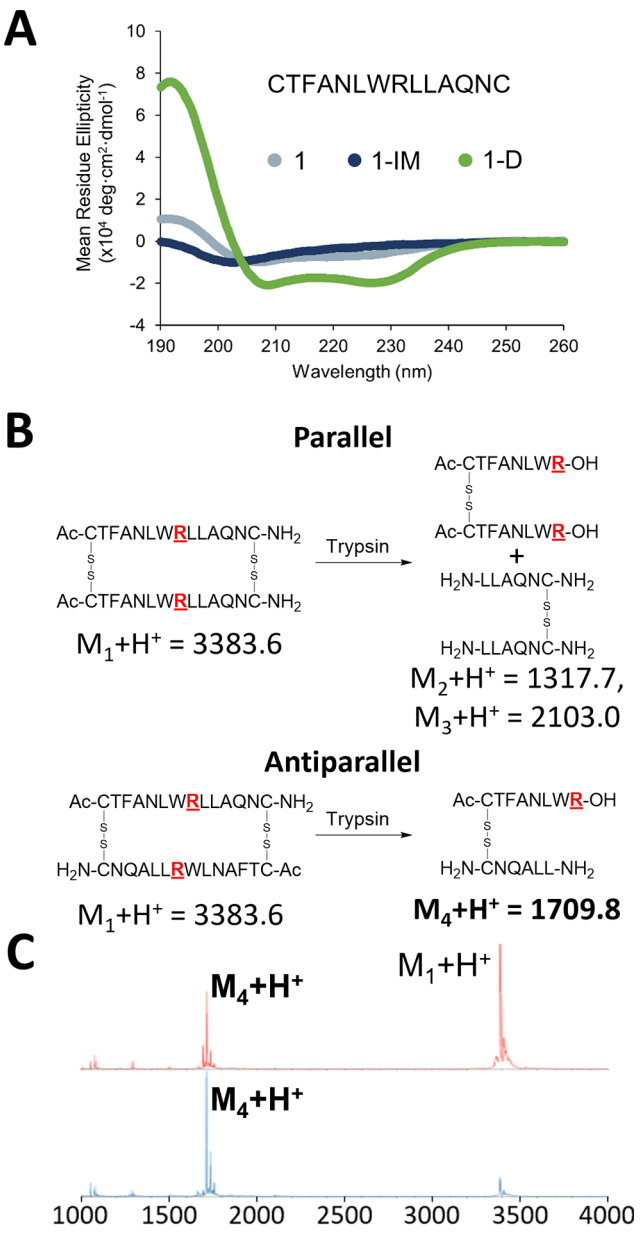

Figure 2. Structure of peptide **1** and its disulfide forms. (A) Circular dichroism spectra of bisthiol **1** (light blue), intramolecular disulfide **1-IM** (dark blue), and disulfide-linked dimer **1-D** (green) at 30 μM peptide concentration in 10 mM sodium phosphate, pH 7.6 at 25 °C. Estimated 5−10% error in calculated mean residue ellipticities for each peptide from at least two replicates per peptide. (B) Predicted products of two possible dimer orientations of **1**. (C) MALDI mass spectra of trypsin digest of **1-D**, showing only the product consistent with an antiparallel dimer after 1 h (red) or 24 h (blue).

residues are known to promote helix folding,[33,34] so this peptide was expected to react and fold similarly to **1**, i.e., dimerize under nondenaturing conditions and exhibit α-helical folding in the bisthiol and dimeric states. Peptide **3**, CTFANLWRGLAQNC, introduces a helix-breaking glycine in the middle of the sequence (L25G using standard p53 numbering), so if reaction selectivity is dependent on α-helical folding under nondenaturing conditions, peptide **3** is not expected to form dimers.

Reaction results were consistent with these predictions. Bisthiol peptide **2** exhibited the same reactivity as **1**: the

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peptide dimer **2-D** was the dominant product upon oxidation under nondenaturing conditions while intramolecular disulfide **2-IM** was the dominant product upon oxidation under denaturing conditions (Table 2). Substitution of the two alanine residues in **1** for two Aib residues in **2** led to a slight increase in α-helical folding for peptide **2** (~35%, Figure S6A). Intramolecular disulfide **2-IM** is nonhelical, and dimer **2-D** is more helical than **2** (~50%, Figure 3A).

For bisthiol peptide **3**, oxidation under nondenaturing and denaturing conditions yielded predominantly the intramolecular disulfide **3-IM** with almost no evidence of dimerization (Table 2). Inclusion of 40% TFE, which increases the level of helical folding of peptide **3** (Figure S6B), did not lead to significant dimer formation in the aqueous DMSO reaction (~5%) with approximately 50% **3-IM** formed and significant quantities of starting material **3** remaining. We speculate that TFE, although it increases α-helical folding of peptide **3**, also reduces hydrophobic interactions between peptides and thus inhibits dimerization. This observation suggests that some amount of α-helical folding in aqueous buffer is essential for disulfide dimerization, though we cannot rule out the potential contribution of reduced overall hydrophobicity from the L25G substitution.

To further explore the unexpected propensity of **1** to form antiparallel dimers and the broader utility of this approach for peptide dimerization, we performed experiments with two sets of peptides (Table 3). In the first set, we varied the positions of the phenylalanine (F), tryptophan (W), and leucine (L) residues, which lie in register with the C-terminal cysteine and are critical for the interaction of p53-derived peptides with Mdm2.[23] Peptides **1-scr1** (CT**L**ANL**F**RL**W**AQNC) and **1-scr2** (CT**W**ANL**L**RL**F**AQNC) are both partially α-helical (Figure S7). Both peptides exclusively formed intramolecular disulfide species under denaturing conditions. Peptide **1-scr2** showed the same preference as **1** for antiparallel dimerization under nondenaturing conditions (90+% yield of antiparallel dimer, Figure 3B). On the other hand, oxidation of **1-scr1** under nondenaturing conditions showed a mixture of products,

Table 2. Effect of Peptide Sequence on Disulfide Bond Formation/Oligomerization

		percent yield[a]		
peptide	conditions	bisthiol[b]	IM[c]	dimer[d]
1	N[e]	1	0	97
	D[f]	8	86	0
2	N[e]	0	0	83
	D[f]	0	77	0
3	N[e]	0	83	1
	D[f]	0	83	1

[a]Yields calculated from integration of HPLC chromatograms for at least two replicates each (all standard deviations <5%). [b]Initial bisthiol peptide. [c]Intramolecular disulfide. [d]Disulfide-linked dimer. [e]Nondenaturing (N): 20% DMSO in 5.5 mM NH_4HCO_3, pH 6.0, 25 °C, 24 h. [f]Denaturing (D): 10 equiv disulfiram (DSF), 5.0 M guanidinium chloride, pH 7.2, 37 °C, 10 min.

Table 3. Peptide Numbering and Sequences[a]

peptide	sequence
1	Ac-CT**F**ANL**W**RL**L**AQNC-NH_2
1-scr1	Ac-CT**L**ANL**F**RL**W**AQNC-NH_2
1-scr2	Ac-CT**W**ANL**L**RL**F**AQNC-NH_2
2	Ac-CT**F***B*NL**W**RL**L***B*QNC-NH_2
3	Ac-CT**F**ANL**W**R*G*L**L**AQNC-NH_2
4	Ac-CT*E**NL**W**RL**L**E**QNC-NH_2
Hif1	Ac-CE**L**LRA**L**DQ**V**NWAC-NH_2
Bak1	Ac-CQ**V**GRQ**L**AW**I**GDEC-NH_2

[a]Underlined residues are in helical register with the C-terminal cysteine. Italicized residues in peptides **2**–**4** emphasize differences from those of peptide **1**. B = α-aminoisobutyric acid; E* indicates glutamate residues crosslinked with 1,3-diaminopropane.[47]

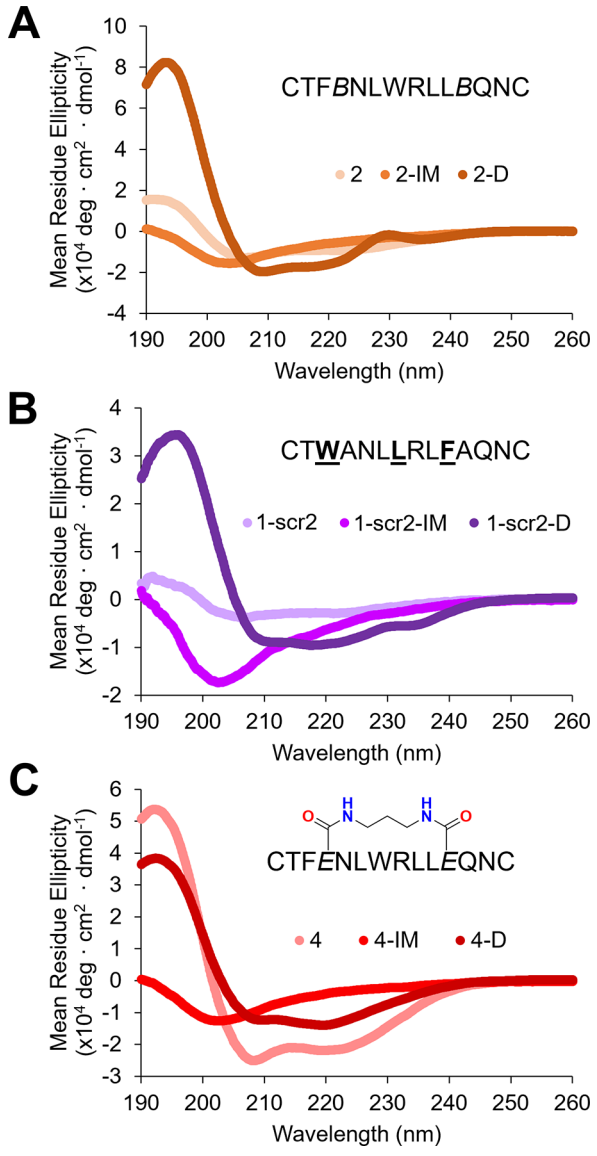

Figure 3. Folding of peptide **1** analogs. (A) Circular dichroism spectra of bisthiol **2** (light orange), intramolecular disulfide **2-IM** (orange), and disulfide-linked dimer **2-D** (dark orange). (B) Circular dichroism spectra of **1-scr2** (light purple), intramolecular disulfide **1-scr2-IM** (purple), and disulfide-linked dimer **1-scr2-D** (dark purple). Residues rearranged from peptide **1** are underlined. (C) Circular dichroism spectra of **4** (light red), intramolecular disulfide **4-IM** (red), and disulfide-linked dimer **4-D** (dark red). All spectra were collected at a 30 μM peptide concentration in 10 mM sodium phosphate, at pH 7.6 at 25 °C. Estimated 5–10% error in calculated mean residue ellipticities for each peptide from at least two replicates per peptide.

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including two distinct dimeric species. In this case, the antiparallel dimer was a minor product (11%). The putative parallel dimer was the major product (53% by HPLC integration) but could not be isolated due to coelution with other species.

These data led us to a preliminary model for dimerization based not only on partial α-helical folding under aqueous conditions but also on interactions between aromatic residues at the helix−helix interface, with tryptophan−aromatic interactions favoring antiparallel dimerization (Figure 4). The

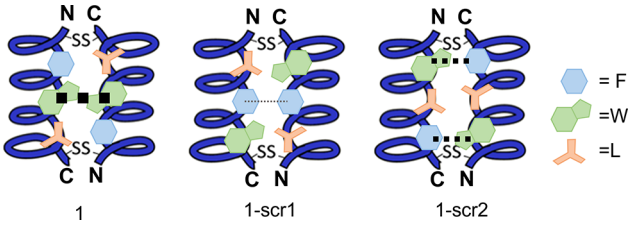

Figure 4. Proposed model for antiparallel peptide dimerization. The FWL triad in peptide **1** is in helical register with the C-terminal cysteine, such that antiparallel dimerization introduces (and may be mediated by) a W/W interaction at the helix−helix interface. Similarly, a pair of W/F interactions can form and promote an antiparallel dimer for **1-scr2**. An F/F interaction is present in **1-scr1**, but a lack of tryptophan−aromatic interactions reduces the bias for antiparallel dimerization.

presence of aromatic−aromatic interactions is consistent with CD spectra, which display atypical minima at 225−235 nm. CD spectral features in the same wavelength range are characteristic of widely known aromatic−aromatic interactions that stabilize antiparallel β-sheets[19,35,36] and have been described more recently in various helix−helix interfaces.[37]

Dependence of dimerization on both α-helical folding and aromatic−aromatic interactions at the dimerization interface was supported by the inability of our second set of peptides to form dimers. These peptides were derived from other protein−protein interactions. One peptide, *C*E**L**LRA**L**DQ**V**NWA*C*, was derived from the C-terminal transactivation domain of hypoxia-inducible factor 1 (Hif1) and adopts an α-helical structure when bound to the transcriptional coactivator p300/CBP.[38,39] A second peptide, *C*Q**V**GRQ**L**AW**I**GDE*C*, was derived from the Bcl-2 homologous antagonist/killer (Bak) BH3 domain, which adopts an α-helix when bound to Bcl-xL.[40] Both peptides were designed following principles identical to those of peptide **1**: *i*-to-*i*+13 spacing between terminal cysteines (italics) and a constellation of hydrophobic residues in helical register with the C-terminal cysteine (bold and underlined). Unlike peptide **1**, the hydrophobic residues were a mixture of leucine, valine, and isoleucine, which are more typical of helix−helix interfaces.[41,42] The CD spectrum for the linear Hif1-derived peptide suggests partial α-helical folding, but oxidation under nondenaturing conditions yielded only a nonhelical intramolecular disulfide species (Figure S8A). The Bak1 peptide, though α-helical when bound to Bcl-xL, did not display α-helical folding as a linear peptide and remained nonhelical after oxidation to an intramolecular disulfide species (Figure S8B). Thus, although the Hif1 peptide displays partial α-helical folding, a lack of aromatic residues in the helical register with the C-terminal cysteine supports our model that both α-helical folding and aromatic−aromatic interactions are necessary for dimerization.

Observing these effects of amino acid sequence on dimerization, we were curious to evaluate whether the disulfide oxidation reactions were compatible with other common cross-linking approaches to increase peptides' α-helical folding. Stabilization of linear peptide folding into an α-helical conformation has proven advantageous for targeting a wide array of macromolecules, including proteins and nucleic acids.[43,44] The terminal cysteine placement allowed us to evaluate three different *i*-to-*i*+7 cross-linking approaches: (1) ring-closing olefin metathesis,[45] (2) bisthiol alkylation,[46] and (3) bis-lactam formation.[47] In each case, we replaced both alanine residues in peptide **1** with appropriate residues for cross-linking (general sequence: CTFX^{1}NLWRLLX^{2}QNC, detailed synthesis described in the Supporting Information). Though all cross-linked products were successfully synthesized, the products from olefin metathesis and bisthiol alkylation were not oxidized and further characterized due to poor solubility and poor yield, respectively.

Evaluation of the *i*-to-*i*+7 bis-lactam cross-linked peptide **4** demonstrated compatibility with both disulfide oxidation approaches. As observed with peptide **1**, oxidation under nondenaturing conditions produced dimers while oxidation under denaturing conditions produced an intramolecular disulfide species. CD analysis reveals significant stabilization of α-helical folding for the linear peptide (80−85%, Figure 3C). The intramolecular disulfide species **4-IM** is nonhelical, and the disulfide dimer **4-D** is α-helical. Interestingly, **4-D** is less α-helical than the linear bisthiol peptide **4**, suggesting that dimerization may involve some helical distortion. This observation is consistent with the fact that the N-terminal cysteine is not in helical register with the C-terminal cysteine, requiring some helical distortion to allow both disulfide bonds to form.

In conclusion, we have identified a facile synthetic approach to transform the structure of individual peptides with N- and C-terminal cysteines through intra- or intermolecular disulfide formation. Intramolecular disulfide species can be synthesized under denaturing conditions in an apparently sequence-independent fashion. On the other hand, disulfide dimerization is high-yielding under nondenaturing conditions but highly dependent on both partial helical folding of the linear bisthiol peptide and tryptophan−aromatic interactions at the dimerization interface. Both intramolecular and dimeric disulfide species are more resistant to proteolysis than the linear precursor but are easily reduced to restore the linear precursor. Because the cysteines are at the peptide termini, this approach is compatible with existing peptide cross-linking approaches[43] to stabilize α-helical structure. This approach thus facilitates experiments to understand how different conformations of a single peptide sequence interact with biological targets including proteins, nucleic acids, and lipid membranes. Conformational control also provides a potentially useful approach to study helix-to-sheet structural transitions in aggregation diseases.

MATERIALS AND METHODS

General Information. Commercially purchased solvents and reagents were used without further purification. Nα-Fmoc-protected amino acids and peptide synthesis reagents were purchased from Advanced ChemTech, ChemImpex International, Oakwood Chemical, and Gyros Protein Technologies. Peptides were synthesized manually or using a Gyros Protein Technologies PurePep Chorus synthesizer. Peptides were purified on preparative C_{18} columns using

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reversed-phase high-performance liquid chromatography (RP-HPLC) on a Shimadzu Nexera HPLC system using gradients of water and acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA). Peptide purity was evaluated by analytical HPLC using a Luna 5 μm C18(2) column (150 × 4.6 mm) on an Agilent 1100 HPLC system with a flow rate of 0.3 mL/min (gradient: 5−95% solvent B over 30 min, solvent A = 0.1% TFA, B = 95% ACN, 5% water, 0.1% TFA). High-resolution mass spectrometry data were collected on a Shimadzu MALDI-8020 Benchtop MALDI-TOF mass spectrometer using α-cyano-4-hydroxycinnamic acid as the matrix. Proteinase K (P8107S) was purchased from New England Biolabs. Mass spectrometry grade trypsin was purchased from Fisher (PI90057).

Peptide Synthesis. All peptides were synthesized following standard Fmoc solid-phase approaches on Rink MBHA resin. All peptides were synthesized on a high-loading resin (0.62 mmol/g resin) except in cases of on-resin cyclization. In these cases, low-loading resin (0.31 mmol/g resin) was used instead. All peptides were globally deprotected and cleaved from resin by incubation with Reagent K (82.5% TFA, 5% water, 5% thioanisole, 5% (w/v) phenol, and 2.5% 1,2-ethanedithiol) for 2 h at 25 °C. After filtration, rotary evaporation of TFA and precipitation with diethyl ether yielded the crude peptide. Full peptide synthesis details are provided in the Supporting Information.

Thiol Oxidation Reactions. Nondenaturing thiol oxidation reactions were performed by sequentially adding 0.25 mL of a 100 mM NH_4HCO_3 aqueous buffer at pH 6.0 and 0.9 mL of DMSO to 3.35 mL of peptide in water (5 mg H_2O, 0.883 mM) to produce a solution with final concentrations of 0.66 mM peptide, 20% DMSO, and 5.5 mM NH_4HCO_3 at pH 6.0.[7] The mixture was reacted at RT for 24 h. Denaturing thiol oxidation reactions were performed by sequentially adding 3.35 mL of 6.0 M GdCl (pH 7.2) and 0.15 mL of 10 mg mL^{-1} disulfiram (DSF) in ACN (10 equiv) to 0.5 mL of 1.0 mM peptide in water to produce a solution with final concentrations of 5.0 M GdCl, 0.375 mg mL^{-1} DSF, and 0.125 mM peptide.[8,9] The mixture was placed in a 37 °C water bath and reacted for 10 min. All thiol oxidation reaction mixtures were purified after the final reaction time by RP-HPLC with a gradient of 15−60% ACN over 30 min.

Circular Dichroism. Circular dichroism spectra were acquired using a Jasco J-1500 CD spectrometer at a concentration of 30 μM in 10 mM sodium phosphate (pH 7.6) in a 0.1 cm path length cell.

Trypsin Digestion. Trypsin digestion samples were prepared by combining 50 μL of 300 μM peptide in water, 200 μL of 125 μM phosphate buffer (pH 7.2), and 50 μL of 1.2 μM trypsin in phosphate buffer at 37 °C. Mass spectrometry data were collected from 1 μL aliquots taken from the reaction mixture after 1, 2, 4, and 24 h.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.3c00268.

Experimental procedures, peptide characterization data (PDF)

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Notes

The authors declare no competing financial interest.

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