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Disulfide-Mediated β-Strand Dimers: Hyperstable β-Sheets Lacking Tertiary Interactions and Turns

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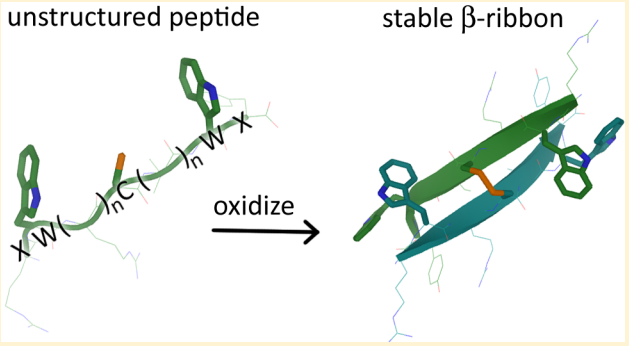
Supporting Information

ABSTRACT: Disulfide bonds between cysteine residues are essential to the structure and folding of many proteins. Yet their role in the design of structured peptides and proteins has frequently been limited to use as intrachain covalent staples that reinforce existing structure or induce knot-like conformations. In β-hairpins, their placement at non-H-bonding positions across antiparallel strands has proven useful for achieving fully folded positive controls. Here we report a new class of designed β-sheet peptide dimers with strand-central disulfides as a key element. We have found that the mere presence of a disulfide bond near the middle of a short peptide chain is sufficient to nucleate some antiparallel β-sheet structure; addition of β-capping units and other favorable cross-strand interactions yield hyperstable sheets. Strand-central cystines were found to be superior to the best designed reversing turns in terms of nucleating β-sheet structure formation. We have explored the limitations and possibilities of this technique (the use of disulfides as sheet nucleators), and we provide a set of rules and rationales for the application and further design of disulfide-tethered "turnless" β-sheets.



INTRODUCTION

β-hairpin peptides have proven useful as model systems for investigating the principles of protein folding and design and as scaffolds for side chain and loop display. However, outside the context of tertiary protein folds, β-hairpins must contain certain obligatory stabilizing features in order to maintain a well-populated folded state. Folding of isolated hairpins requires either a very tight nucleating turn or highly optimized β-strands, typically with high-as-possible β-propensities and cross-strand Trp/Trp pairs. This limits the diversity of designable β-sheet structures.

Disulfide bonds can link adjacent antiparallel β-strands in proteins, but the dihedral angle preferences of disulfides constrain their use.[1−3] Specifically, disulfide bonds are only energetically favorable if the Cys residues are positioned at non-H-bonded (NHB) cross-strand sites.[4−6] Outside of protein contexts, disulfides can be used to staple together otherwise poorly folded hairpins; though the same rule applies: the Cys residues must be introduced at NHB sites. Properly positioned disulfide bonds have been used to cyclize peptide chains and create fully folded controls for β-hairpin fold-population studies.[5,7−9] They also stabilize and rigidify the natural β-hairpin motifs of antimicrobial peptides,[10−14] where multiple cross-strand disulfides are often found in a single β-hairpin. Of these examples, there are cases (e.g., protegrin)[13] where disulfides "rescue" β-hairpins which, due to their poor reversing turns or repulsive cross-strand Arg/Lys residues, are poorly folded when the disulfides are reduced. This begs the question: if poor turns are allowed when this covalent strategy is used, then are turns even necessary? Can small antiparallel β-sheets be brought together as structured disulfide dimers?

Though we feel our diverse array of turn-free β-sheet constructs provides a satisfactory "yes" to the above question, we would be remiss to neglect some recent designs employing disulfide staples to form symmetric β-sheet dimers. For example, the Balaram group designed a C2-symmetric eight-stranded sheet held together by a central cross-strand NHB disulfide[15] (PDB id 1JY4, 1JY6), and the Nowick group has reported stabilization of a cyclic β-sheet template on homodimerization via formation of twin disulfides at the ends of the sheet.[16] The Waters group has shown that polyproline recognition by Trp-rich hairpins can be improved through dimerization at strand-terminal Cys residues.[17] However, these and other examples consist of independently folded β-sheets bound together as dimers, not natively unfolded strands which form two-stranded sheets on oxidative dimerization. Until recently, the closest approaches to designed, disulfide-nucleated β-sheet were the dimer reported by Cashman et al.,[18] which adopted a stable fold only in organic solvents, and the hexameric bundle of β-strands representing the disulfide-bound portion of the prion protein.[19] We recently reported[20,21] two-strand oxidation-dependent homodimeric sheets, presenting them as ideal test systems for evaluating the extent of

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stabilization and end-fraying reduction resulting from our β-capping motifs. We have yet to give a detailed accounting of design considerations and the importance of the disulfide in these constructs. We now seek to present a breadth of examples of this useful class of peptides and lay down the rules for disulfide-mediated β-sheet formation.

METHODS

Synthesis. All peptides were synthesized on a CEM Liberty Blue synthesizer, using standard Fmoc solid-phase peptide synthesis. Preloaded Fmoc-protected Wang and unloaded Rink amide resins were used for synthesis. Peptides were cleaved from resin (0.1 mmol) using a cocktail of trifluoroacetic acid:triisopropylsilane:water (38:1:1, 9.5:0.25:0.25 mL) for 1.5 h. The resulting filtrate was concentrated *in vacuo*, crashed out, and washed with cold (−20 °C) diethyl ether, giving the crude peptide. After drying the peptide, the pellet was dissolved in ∼8 mL water and purified using RP-HPLC (Varian ProStar 220 HPLC, Agilent 21.2 × 50 mm C18 column, 10 mL/min, elutent A: water with 0.1% TFA, elutent B: acetonitrile with 0.085% TFA), using a gradient of 10−50% B over 19 min. The resulting product was dried on a rotovap and oxidized via dissolution in a minimal amount of DMSO (0.5 mL), diluted with water (1 mL), and let stand for 24 h at room temperature. After additionally diluting with water (3.5 mL) the solution was repurified via HPLC using the same method to gauge the relative proportion, if any, of nonoxidized peaks. (Oxidized peaks typically eluted before nonoxidized peaks, presumably due to folding and increased hydrophobic burial.) Peaks were visualized at 215 and 280 nm with verification by mass spectrometry (Bruker Esquire ion trap with ESI ionization). The concentrated fractions were then lyophilized resulting in the purified peptide. If disulfide formation did not proceed using the previous procedure, the dry peptide was dissolved in minimal amount of DMSO (0.5 mL) and diluted in 1 M HCl (1 mL) and left for 24 h. Higher-order assembly (e.g., D2-symmetric dimers of dimers) is a spontaneous process and not a result of covalent modifications or other chemical syntheses steps. Most oxidized dimers in this study showed little evidence for higher order assembly, though they had some propensity to form aggregates or hydrogels in their reduced state.

NMR Spectroscopy. NMR samples were made at ∼1 mM in 20 mM potassium phosphate buffer with 10% D_2O and an internal standard of sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). Full ^1H spectra assignments were made using peptide backbone connectivities determined by 2D TOCSY and NOESY experiments taken on either Bruker DRX 500 MHz or AV 700 MHz spectrometers.

Structuring induced chemical shift changes were analyzed as chemical shift deviations (CSDs, the observed shifts minus the coil reference values) using the CSDb algorithm (andersenlab.chem.washington.edu/CSDb/about.php) as previously described.[22,23] Converting CSDs to fold stability data (χ_F, the fraction folding) requires reference CSDs for the folded state. The CSDs of $(CH_3CH_2CO\text{-}WTTVCIRKWTGPK\text{-}NH_2)_2$, which is 98.5% folded based on H/D exchange data at 280 K,[20] served this function for most of the systems herein. More details appear in the Supporting Information.

CD Spectroscopy. Stock solutions of approximately 200 μM peptide concentration were prepared using 50 mM aqueous pH 7.0 phosphate buffer. Accurate concentrations were determined by UV spectroscopy assuming the standard molar absorptivities for the Trp and Tyr residues present. The samples were typically diluted to obtain ca. 30 μM polypeptide solutions, with spectra recorded on a Jasco J720 spectropolarimeter using 0.10 cm path length cells over a UV range of 190−270 nm as previously described.[24−26] For high-concentration (>1 mM) experiments, a 0.01 cm cell was used. In melting studies, temperatures ranged from 5 to 95 °C in increments of 5° or 10°. For CD melting temperatures, the folded fraction (χ_F) was determined by defining the temperature-dependent CD signal of the unfolded and folded states and assuming a linear χ_F relationship for signals between the two lines. The CD spectrum of the unfolded state is expected to be sequence independent at 228 nm, the position of the fold-diagnostic exciton couplet.

RESULTS

Single Disulfide Homodimers. It has long been known that disulfide bonds are favored at the non-H-bonded positions of antiparallel β-sheets.[4−6] Until the present studies it had not been established that disulfide formation can nucleate antiparallel association of "turnless" strands into isolated β-sheet structures. In control peptides for some of our β-cap studies, we observed the nucleation of β-structure centered on a non-H-bonded cystine. This phenomenon was observed even for very small, symmetric peptide dimers with central cystine residues. For example, the oxidized disulfide form of $(KKVCITT)_2$ displays CSDs from random coil norms that indicate antiparallel β-sheet formation with the folded state ca. 65% populated ($\chi_F \approx 0.65$) at 280 K and pH 7 and $\chi_F \approx 0.4$ at pH 2. The pH effect suggests that the terminal salt bridges provide additional stabilization. When the β-strands flanking the disulfide bond are improved or lengthened, the resulting sheet structures can become remarkably stable, particularly with β-capping motifs[20,21,27] (Trp/Trp pairs at strand-terminal NHB positions and their supporting interactions) present. As dimeric structures the stabilizing caps appear twice in one β-sheet, and the net fold-stabilization they provide is doubled.

Like $(KKVCITT)_2$, peptide $(KWRCIWD)_2$ is a seven residue peptide dimerized by a central cystine. However, it incorporates a version of the Coulombic β-cap[21] at both ends of the homodimer, and the β-sheet structure it adopts is much more stable ($\Delta\Delta G_U > 15$ kJ/mol). It is effectively 100% folded at 280 K and has a melting point of 70 °C based on the temperature-dependent circular dichroism (CD) spectrum (see Figure S7, Supporting Information, for full CD melt). This is remarkable for a peptide synthesized with just seven natural amino acids. The two β-caps not only impart great fold-stability but also provide useful spectroscopic diagnostics of fold formation: a high amplitude exciton couplet in the CD (max: 228 nm) and an Hε3 proton for the "edge" Trp shifted upfield >2.2 ppm (at 100% folding). Some related examples of hyperstable β-sheet dimers, or β-ribbons, are collected in Table 1.

The fold stability data in Table 1 indicates that the β-capping strategy is orthogonal to disulfide dimerization; these techniques work in combination but do not require each other. For example, compare peptide Pr-WTTVCIRKWTGP-NH_2 vs +WTTVCIRKWTGP-NH_2; the latter has disabled β-caps, as it lacks the essential N-terminal alkanoyl function.[20] Though it loses ca. 12 kJ/mol of stability, it remains 70% folded at 280 K. More dramatic is the case of peptide $(KWTTHCHRKWT)_2$ which is stabilized by superior, hydrophilic caps. Hydrophilic capping units maintain the essential cross-strand Trp/Trp pair but reinforce it with a terminal salt bridge instead of an indole-amide H-bond with the $i + 2$ Gly.[21] This peptide is ≥96% folded at 280 K (pH 8.0), while its isomer with C-terminal W and T residues swapped (thus abolishing the β-caps) is almost entirely unstructured under the same conditions. Uncapped β-strand dimers with central disulfides were observed to be at least ∼50% folded when rich in β-branched residues (e.g., entries 2 and 3 from Table 1); we presume that the near-zero-fold population of this cap-abolished dimer is due to the poor β-propensity of the disulfide-flanking histidine residues.

Our hyperstable β-ribbon constructs take two forms, (alkanoyl group)-$W(X)_{n1}C(X)_{n2}WTG\cdots$ (designated as a hydrophobic cap) and $ZW(X)_{n1}C(X)_{n2}WZ$ (the hydrophilic cap, where Z can be any residue, but ideally with opposite

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Table 1. Cys-Containing Peptides Displaying Disulfide-Mediated β-Sheet Formation

Sequence	χ_F	T_M (CD)
VCI	0.20	
KKTCTTT	0.52	
KKVCITT	0.72	
PWVCKHT	0.88	
KWRCIWD	≥0.98	69
HWVCIWR	≥0.98	87
RWRCIWY	≥0.98	
HWHCIWD	≥0.97	
KWRTVCIRTWE	≥0.98 [a]	75
KWRTIKVCITKRTWE	0.99	
Pr-WVCKWTGPK-NH_2	0.97	72
WVCKWTGPK-NH_2	0.17	
Pr-WTTVCIRKWTGP-NH_2	0.985 [b]	81
WTTVCIRKWTGP-NH_2	0.74 [b]	
Bz-WHTHCIRTWTGP-NH_2	0.89	
Bz-WITKCIRKWTGKK	0.94	78
KWTTHCHRKWT	0.96	
KWTTHCHRKTW	0.06	
KWTTHCHRKWA	0.93	70
KWTTHCHRKWA *REDUCED*	0.00	

Pr-: N-terminal propanoyl, Bz-: N-terminal benzoyl. All values are for the oxidized species, except as noted. The fraction folded values are based on NMR data for pH 6−8 at 280 K. All reduced species show no evidence, whatsoever, of folding by NMR and CD spectroscopy. Complete NMR chemical shift data can be found in the Supporting Information for peptides KWRCIWD and HWVCIWR (Table S2, Supporting Information) and KWTTHCHRKXX (the last four entries of this table; found in Table S6, Supporting Information). [a]From ref 20 [b]From ref 19; determined with precision by amide H/D exchange methods.

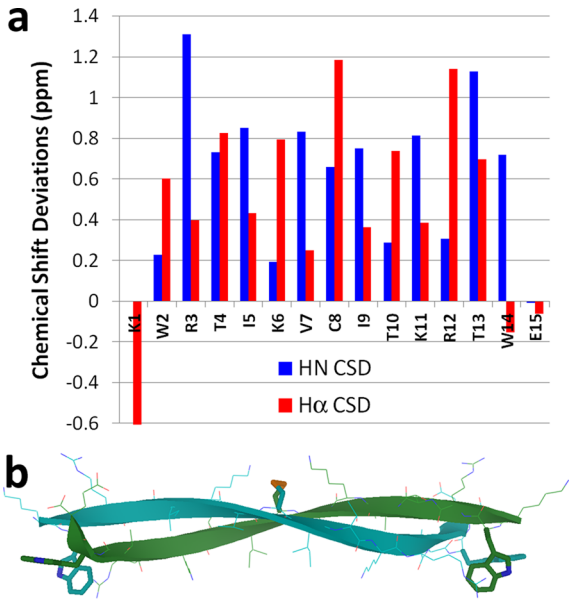

Figure 1. (a) CSDs of a long disulfide dimer, (KWRTIKV-**C**ITKRTWE)$_2$. The expected β-sheet periodicity is observed: inward pointing Hα and H_N protons are strongly shifted downfield from expected-coil values (0.7−1.3 ppm), while outward-pointing protons are less shifted, at 0.3−0.5 ppm. (b) Example structure of the disulfide dimer (β-ribbon) from (a) (KWRTIKV**C**ITKRTWE)$_2$, with the key disulfide and capping Trp residues highlighted as sticks. (This structure is supported by and consistent with NMR data, including all expected cross-strand NOEs, but not explicitly generated via annealing to NOE distance constraints. This structure was generated using a standalone version of Foldit,[28] with key NOEs and known H-bonds included as restraints during manual folding. A complete table of NOE restraints appear in Table S7, Supporting Information.)

charges at the two ends).[21] The alkanoyl group can be virtually any small molecule,[26] and X can be any residue(s) other than proline. However, *n*1 must equal *n*2, and the value must be an odd number. This is necessary to maintain the disulfide at the central position of the β-sheet and keep it in-register with the capping tryptophan residues. The disulfide and both cross-strand Trp/Trp pairs must be at NHB positions; as a result, constructs of the form ZW(X)**C**(X)WZ (hydrophilic cap) are limited to β-sheet lengths that satisfy a 3 + 4*n* formula, 7, 11, 15, 19, etc. are possible strand lengths.

These peptides are contiguous β-sheets, analogous to long hairpins but with no turn at either end. The longer β-sheet constructs of this form have some local distortion (a change in the twist) at the disulfide, as evidenced by a slight anomaly in the CSD patterns (Figure 5 provides the most extreme example), but overall a defined β-sheet structure is maintained. See Figure 1.

Comparing the β-Sheet Nucleation Strengths of Disulfides versus an Optimized Turn Sequence. Previous work aimed at designing small, stable β-sheet constructs typically employed turn sequences including D-AAs to favor chain reversal. Among these, the heterochiral diproline unit (D-Pro L-Pro[29−31]) is probably the most effective. This sequence favors a type II′ β-turn and provides exceptional entropy-based stabilization for β-hairpins; only a handful of exotic turn-nucleating small molecules are slightly better.[32,33] All turn sequences composed only of L-AAs and glycine are significantly poorer. We have now established that a strand-central cystine residue has a higher β-structure nucleating propensity than the heterochiral diproline unit (Table 2).

The comparison begins with peptides KWRWRTR-**C**-RKIKRWY and KWRWRTR-pP-RKIKRWY, identical strands with different linkers. The strands are favorably capped, but the intervening residues have only middling β-propensities; in addition, they are exceptionally rich in Arg and Lys, with two repulsive cross-strand R/R interactions per strand pairing. This serves to alleviate any potential solubility problems and reduces the fold populations to measurable values despite the presence of the β-cap (which provides the spectroscopic diagnostics for measuring χ_F). Incidentally, these constructs strongly resemble antimicrobial peptides and RNA-binding peptides[34] in residue composition.

Peptide "C" was significantly more stable than "pP", despite having one less residue. Thus, when designing stable β-sheets, a single disulfide would appear superior to the best known hairpin-nucleating turn sequence from peptide engineering efforts. However, this is not a perfect comparison for determining raw hairpin nucleation potential, as disulfides can nucleate β-sheet formation in two directions and form a single contiguous sheet double the length of an analogous monomeric hairpin. If considered as separate sheets, the two halves on either side of the central disulfide are cooperative in their folding; the folding of one-half nucleates the other. Therefore, for a direct comparison, the disulfide-bound heterodimer comprised of KWRWRTR**C**R and R**C**RKIKRWY was synthesized and characterized. This species (listed as "half" in Table 2) was found to form a reasonably stable hairpin, though not

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Table 2. Peptides with Identical Strands (KWRWRTR and RKIKRWY) but Different Connecting Units[a]

Name	Sequence	description	T_M (°C, CD)	300 K χ_F (NMR)
C	KWRWRTR--C--RKIKRWY YWRKIKR--C--RTRWRWK	(homodimer)	44	0.95
CIC	KWRWRTR-CIC-RKIKRWY YWRKIKR-CIC-RTRWRWK	(homodimer)	58	0.96
pP	KWRWRTR-$_p$ YWRKIKR-^{p}	(hairpin monomer)	36	0.88
Half	KWRWRTR--CR YWRKIKR--CR	(hairpin-like heterodimer)	24	0.66

[a]All peptides containing Cys are oxidized, with disulfide bonds between strands.

quite so stable as the pP variant (T_M ca. 24 °C vs 36 °C for "pP"). CD data and the method for determining fraction folded appear in the Supporting Information. An NMR comparison of the peptides in Table 2 appears in Figure 2.

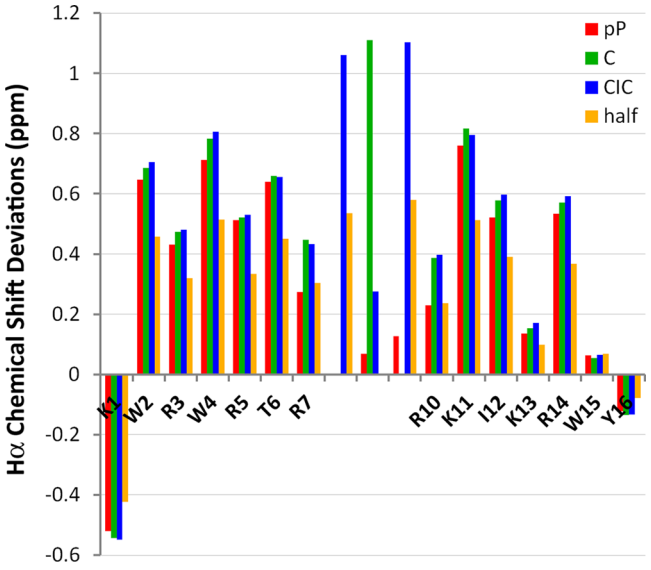

Figure 2. NMR structuring shift comparisons (CSDs) for peptides "C", "pP", "CIC", and "half" (Table 2) at 300 K. The corresponding CD spectral comparisons appear in the Supporting Information.

Paired Disulfides as β-Structure Nucleators. As previously noted, antiparallel β-sheets with disulfide-linked, capped β-strands are limited in their diversity and application by the requirement that all of the Trp and Cys residues must be at NHB strand positions. Thus, many strand lengths and compositions are precluded. Some of these limitations can be lifted by using a CXC sequence rather than a single disulfide. The data in Table 2 illustrate that this can provide even greater β-structure stabilization. Prior studies have established that peptides with CXC sequences can form both antiparallel and parallel homodimeric sheet structures on oxidation and that homodimers are strongly favored over intramolecular disulfide formation.[35]

In our studies, this simple two-disulfide $i + 2$ motif is more redox stable than isolated cystines, largely orthogonal to single-disulfide oxidation and a better β-sheet nucleator. No parallel dimers were observed for peptide "CIC" (Table 2), likely because β-caps do not work on parallel β-sheets, and antiparallel sheets are generally more stable than their parallel counterparts. It also oxidized much more rapidly than its single-Cys analog.

The strong preference for disulfide formation across CXC units leading to self-associated dimers, versus slower disulfide formation for other cysteine residues, opens up convenient biotechnology applications. Specifically, functionalization of free cysteine residues can be carried out on a structured β-sheet held together by a set of $i + 2$ disulfides. For example, we were able to purify RW**C**TK**C**I**C**IRKWE as the partially oxidized dimer after 14 h of air exposure in neutral buffer. Higher order assembly on complete oxidation resulted in a complex mix of soluble, high-molecular-weight covalent oligomers.

Alternative Disulfide Locations and Heterodimers. Disulfide-mediated β-sheets are not inherently limited to homodimers. We expected that heterodimers would be viable targets for design, though there are clearly yield considerations due to competition with homodimer formation. To examine the effects of alternative disulfide linkages on β-sheet formation, we replaced individual cysteine residues of fully folded (when oxidized) parent peptide RWITK**C**I**C**IRKWE with serine, to investigate "off-center" heterodimers (Figure 3).

We were surprised to discover that yields for the oxidative disulfide formation approached 100% heterodimer, with little interference from homodimers with diagonal disulfide linkages. Of note, the heterodimer allows formation of both a NHB cross-strand disulfide and two β-caps. The resulting off-center dimer was fully folded, with a T_M of ca. 95 °C (as observed via CD). Curiously, the chemical shifts were nearly identical for the two strands; there were slight differences for the asymmetric central region, but perfect peak overlap for the terminal (capping) residues.

We also investigated homodimers of these two single-disulfide species, to explore the fold-stabilizing capacity of disulfides bridging either of the nonequivalent diagonal positions. The natural twist of isolated β-sheets staggers the sidechains and favors one diagonal interaction over the other for disulfide formation. For right-hand, twisted β-sheets, sidechains at NHB positions can make close contacts with opposite-strand sidechains in the direction of their own strand's C terminus; this is sufficient, if not optimal, for disulfide formation. But the distances between opposite-strand sidechains toward each others' N terminus are too great for disulfide formation, in β-sheets of standard antiparallel

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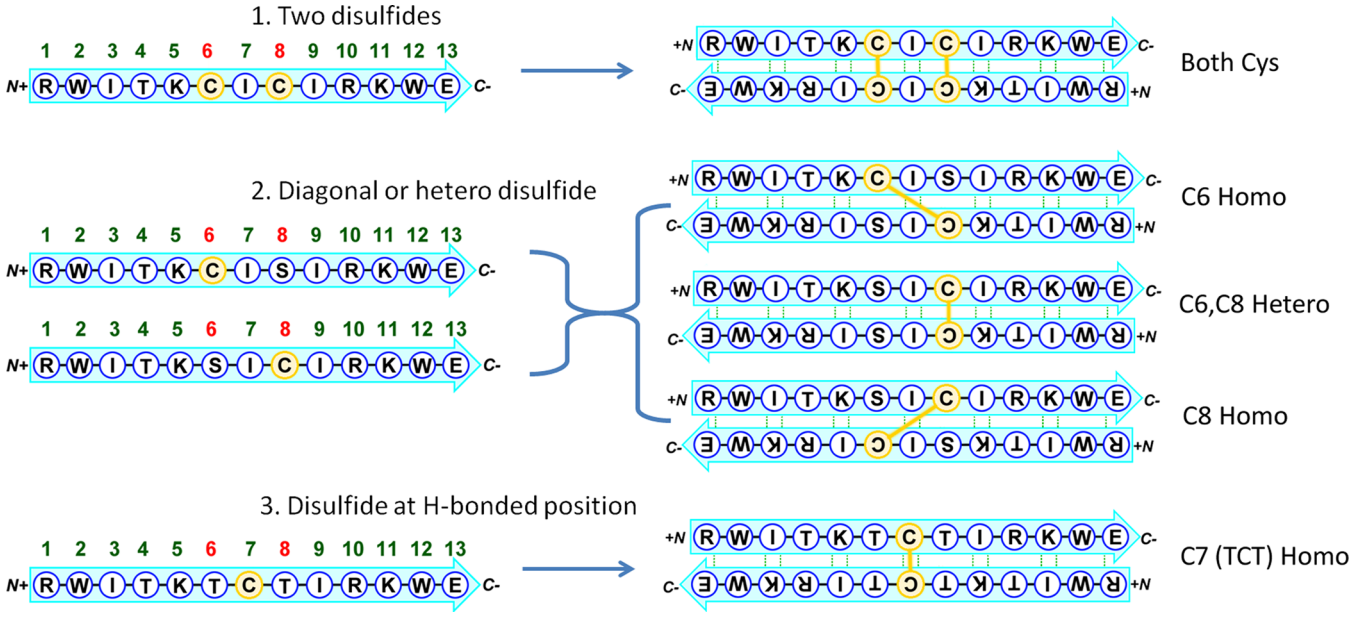

Figure 3. Schematic illustrating the assembly and disulfide positions of the dimeric β-sheet peptides in Table 3.

geometry. Thus, a diagonal disulfide between C6 and C6′ would be compatible with the register of a doubly capped, stable β-sheet, while a potential disulfide between C8 and C8′ would not. The diagonal interactions discussed here, and the β-sheets we used to test diagonally positioned disulfides, are illustrated in Figure 3. There is some literature evidence for β-sheet stabilization by a diagonal disulfide (e.g., antimicrobial peptide androctonin,[36] PDB id 1CZ6) but only near the ends (rather than the middle) of the associated antiparallel β-strands.

We also investigated a nearly identical sequence with the Cys residue at an H-bonded position. (H-bonded cysteines are technically close enough to their cross-strand symmetry pair to form a disulfide bond, but this is unfavorable due to steric features and rotamer preferences.) We changed the Cys-flanking residues to threonines as a compromise between isoleucine and serine.

As expected, the stability order (as established by CD; Figure 4) was "both Cys" (two cross-strand NHB disulfides) > C6,C8 heterodimer (one cross-strand NHB disulfide, off-center) > C6 homodimer (favored diagonal disulfide) > C7 homodimer (cross-strand HB disulfide) > C8 homodimer (disfavored diagonal disulfide). In all cases, strand register was fixed by the β-caps, not the disulfides. This series of peptides clearly illustrates that cross-strand disulfides are considerably more favorable than alternatives, yet even strongly disfavored disulfides (e.g., C7 homo peptide's HB position disulfide) allow themselves to adopt strained conformations rather than alter the register as defined by the strong (but noncovalent) β-capping interactions. The capacity of cross-strand Trp/Trp interactions to "outcompete" disulfides as fold-stabilizers and strand register anchors of β-sheet structures has been observed previously.[37]

Deviations from Canonical Antiparallel β-Sheet Structure Are Observed for Disulfide-Dimerized Strands. Disulfides are well-suited for non-H-bonded positions, but they do impose some additional structural constraints. While the examples in the previous section show they are not always capable of defining their strands' register to achieve a non-H-bonded cross-strand disulfide, they are

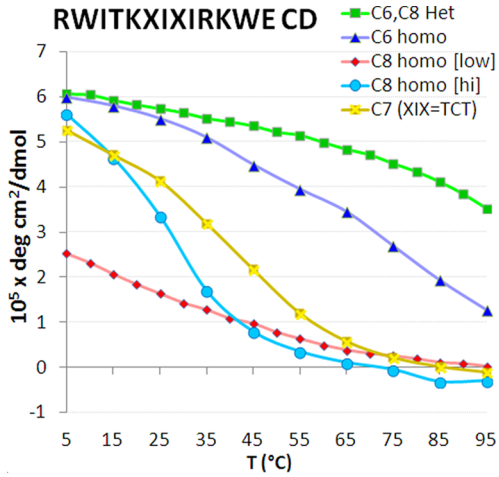

Figure 4. CD melting curves (molar ellipticity measured at the 228 nm maxima of the exciton couplet versus temperature) for the five peptides in Table 3. Peptide C8 homodimer was investigated at both high (1.5 mM) and low (30 μM) concentrations. For the corresponding NMR comparisons see Figure S6, Supporting Information.

nonetheless capable of altering the twist and buckle of their β-sheet. The Varadarajan laboratory[4,38] found that, even at their most-allowed cross-strand NHB positions, disulfide bonds lowered cyst(e)ine Cα distances and caused β-sheets to buckle inward on the disulfide-bearing face. This imposition is expected to be greater for a set of two or more $i + 2$ disulfides.

Typical β-hairpins display a characteristic pattern of chemical shift deviations. Most notably, every second amide proton (the inward-pointing, cross-strand H-bonding protons) exhibits far-downfield (ca. 1 ppm) CSDs as a result of strong H-bonding to the cross-strand carbonyl, and an in-plane alignment between the β-strands. Disulfide-disrupted β-structure can be identified most readily by a change in this pattern of H_N chemical shift deviations. The cyst(e)ine residues have higher than expected chemical shifts for non-H-bonding strand positions, and neighboring (H-bonded) residues are not as downfield as

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expected. H-bonds are presumably still present at these sites, but they may be weakened by strand buckling and/or other distortions required to accommodate the disulfide bond. We have observed that this buckling effect is subtle for lone disulfides and short sheets but becomes increasingly obvious when: (a) the length of the associated β-strands increases, (b) the number of consecutive $i + 2$ disulfides increases, or (c) the neighboring residues (at H-bonded sites) are less bulky (not β-branched). The Supporting Information provides a table of ratios between the H_N CSDs for cysteine and its immediate neighbors; we view this ratio as a diagnostic for disulfide-induced β-strand distortion. An extreme test case, a de novo antimicrobial peptide with a triple-disulfide $(RWKCKCKCKWE)_2$, (three consecutive $i + 2$ disulfides, no β-branched residues, and moderately long β-strands) has an inverted pattern of H_N CSDs. The data are shown in Figure 5.

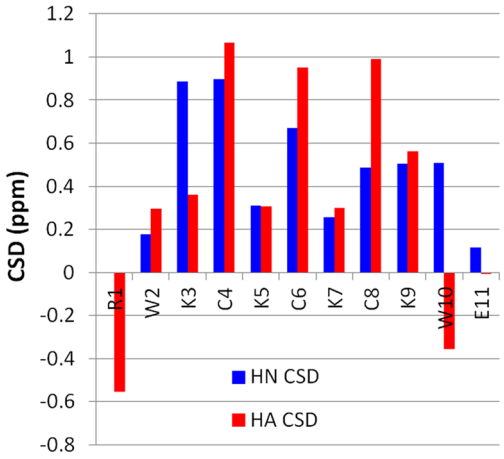

Figure 5. H_N CSDs for peptide $(RWKCKCKCKWE)_2$. The β-sheet is highly distorted, and this manifests as an inverted pattern of H_N CSDs: larger CSDs are observed for the outwardly directed non-H-bonded positions (the disulfide positions at C4, C6, C8) rather than the H-bonded positions (especially the presumably-H-bonded K5 and K7 sites).

Building on the Core Sheet: How to Make Four-Stranded Sheets. Disulfide-dimerized β-sheets would appear to be ideal cores for capture and docking of outer β-strands. Curiously, of our four attempted designs of four-stranded sheets (dimers of β-hairpins sewn together by a disulfide centered on one strand), all but one yielded a stable central core juxtaposed with an unstructured region. We had expected a strong templating effect from the fully folded core strand, as is typical of three-stranded sheets.[39,40] We now conclude that the distortion of the end-capped hub strands (e.g., WXCXW) precludes formation of all but the most favorable β-hairpin dimers. Data for the successful four-stranded sheet motif (sequence: $(RWICRWWDEKSGRWITKKID)_2$) are shown in Figure 6. Further examples appear in the Supporting Information.

Disulfide Stabilization of Antiparallel β-Sheet Structures Does Not Apply When the Disulfide Unit Is Not Located in the β-Strands. Disulfide bonds can impart β-sheet structure better than the best turns, on a per-chain basis. Yet their use in this regard is highly context dependent. The example of RWITKXIXIRKWE (Table 3, Figures 3 and 4) illustrates that, while cross-strand non-H-bonded positions are

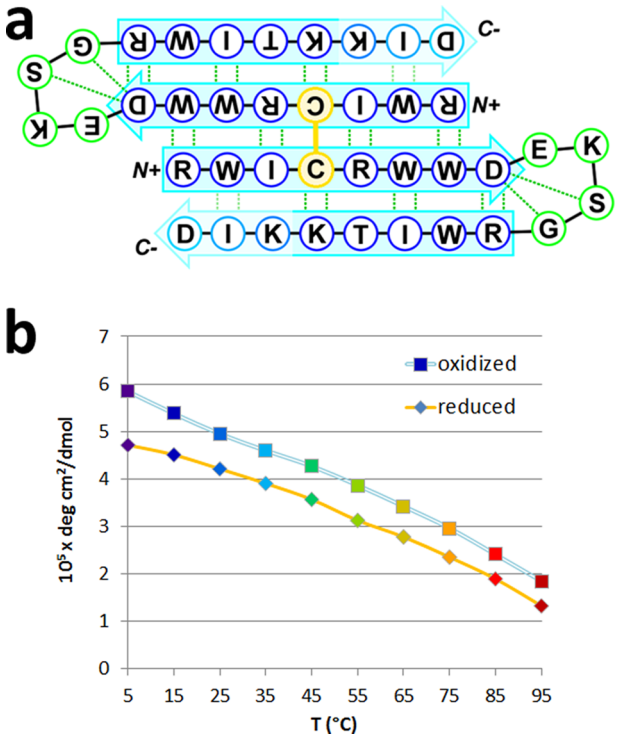

Figure 6. (a) Cartoon image illustrating β–β dimer topology. (b) CD-derived melting plot; intensity vs temperature. (Trp/Trp exciton couplet maxima at 228 nm.) The reduced form still adopts a fully folded hairpin. NMR data (see Supporting Information) suggest that the reduced species remains monomeric and that the intrachain Trp/Trp interaction (present in both oxidized dimer and reduced monomer) produces a stronger exciton couplet than the interchain Trp/Trp (oxidized dimer only) pair.

strongly preferred, this preference is not enough to shift the strand register required by β-caps.

Table 3. RWITXIXIRKWE Peptides: The Effect of Relative Cyst(e)ine Placement

name	sequence	T_m (CD)	280 K χ_F (NMR)
C8 homodimer (bad diagonal)	RWITKSICIRKWE EWKRICISKTIWR	29[a]	0.95[a]
C6 homodimer (good diagonal)	RWITKCISIRKWE EWKRISICKTIWR	72	0.98
C6,C8 hetero (X-strand)	RWITKCISIRKWE EWKRICISIRKWE	~95	0.99
both Cys (2× X-strand)	RWITKCICIRKWE EWKRICICKTIWR	>100	>0.99
C7 (TCT) homo (HB position)	RWITKTCTIRKWE EWKRITCTKTIWR	42	0.88

[a]At high concentration (1 mM), as a soluble oligomer. See the Higher Order Oligomers section (below) for details.

Further counterexamples were sought to help set the boundaries of the β-sheet-enforcing capabilities of disulfides. Peptide RWITVTIGGGGCGGGGKKIRVWE forms an antiparallel β-sheet structure (χ_F = 0.91 at 280 K) even in its reduced form with the nine residue GGGGCGGGG sequence remaining a fully flexible loop devoid of structure. While disulfide formation would not decrease the length of loop that

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would need to be conformationally searched to achieve β-strand association, it can effectively lower loop search times by increasing local concentrations of equivalent strands. Furthermore, if one hypothesizes that disulfides are exceptionally effective nucleators of β-sheet structures, one might expect the flexible loop to overcome the significant entropy barrier imposed by the eight glycine residues and form into a contiguous β-sheet, as shown below:

A RWITVTIGGGG**C**GGGGKKIRVWE **B**

B′ EWVRIKKGGGG**C**GGGGITVTIWR **A′**

As it turns out, the glycine loop region of both the reduced and oxidized forms of this peptide display nearly identical chemical shifts with negligible CSDs; this rules out the possibility shown immediately above. The β-sheet fold population of the oxidized species is somewhat higher (χ_F => 0.97, at 280 K, vs 0.91), but this may be nothing more than a local concentration effect which increases the folding rate. When oxidized, strand A can find both strand B and the equivalent B′ nearby, with which to form a β-sheet. When reduced, strand A has only strand B to pair with. Due to the flexibility of the GGGG**C**GGGG segment, all combinations of AB, A′B, AB′, and A′B′ are essentially equivalent. (See Figure S3, Supporting Information for a schematic of this peptide's potential folding pathways.) Thus, we can delineate the limitations of the disulfide strand nucleation strategy: Disulfides are not magic bullets that will generate local β-sheet structures where no propensity to form sheets is otherwise present. Also, the enhancement of association through a local concentration effect does not appear to be dramatic or essential in cases where folding is possible without dimerization.

Higher Order Oligomers. The use of disulfides to form stable β-sheets has an interesting consequence: Highly charged, amphipathic β-sheets can be constructed with ease. Whereas rows of cross-strand arginines and lysines would normally repel each other and preclude folding, disulfides can force them into proximity. A secondary consequence is that amphipathicity becomes easy to design; β-sheets can have very high net charges and concentrate these charges on one face. This architecture is exploited by many natural antimicrobial peptides, where their high positive charge and lipophilic face enable them to disrupt bacterial membranes. (It helps that disulfides can, in themselves, form a hydrophobic face.[13]) This easy route to amphipathicity can also afford β-sheets capable of oligomerizing while remaining soluble. For example, a disulfide-linked heterodimer of human prion protein fragments (the region surrounding the disulfide bond: VD**C**VNI and EQM**C**IT) was also shown to assemble into soluble trimers-of-dimers.[19]

Some of the peptides in this study were observed to form soluble oligomers—likely dimers-of-dimers—though most evidence was indirect. For example, triple-disulfide homodimer RWK**C**K**C**K**C**KWE has a hydrophilic face (R,K,K,K,K,E) and a hydrophobic face (W,**C**,**C**,**C**,W). Its severe line-broadening (for strand-central residues only) suggests the formation of a noncovalent dimer-of-dimers or some other soluble oligomeric form.

All short dimers of the form $(X\mathbf{W}X\mathbf{C}X\mathbf{W}X)_2$ are amphipathic, with a fully hydrophobic W,**C**,W face regardless of the identities of the other residues. This surface is capable of sticking to a copy of itself and appears to form a D2-symmetric dimer-of-dimers. However, this second oligomerization event is remarkably slow for a noncovalent interaction between small peptides. Two distinct sets of peaks corresponding to two β-sheet conformers of slightly different structure are observed in NMR spectra at high concentrations. Simple models suggest these peaks represent disulfide-swapped structures, wherein the edge-to-edge cross-strand disulfide is exchanged for a pair of face-to-face disulfides in a D2-symmetric tetramer. Tellingly, NOEs are observed between opposite ends of individual indole rings for the isomer responsible for one of the sets of peaks. 1D NMR spectra of a $(X\mathbf{W}X\mathbf{C}X\mathbf{W}X)_2$ peptide at varied concentrations (see Figure S9, Supporting Information) confirms that one set of peaks represent a higher-order species present only at the higher concentration. A proposed model that satisfies the CSDs and NOEs is shown in Figure 7.

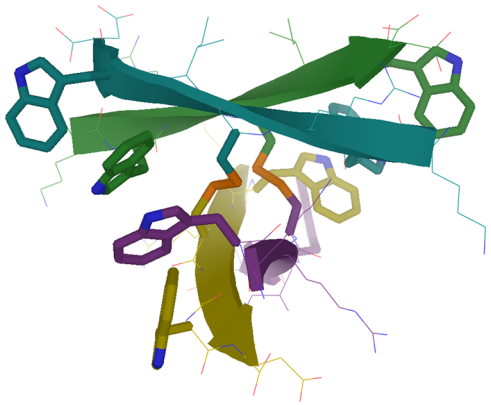

Figure 7. A proposed structure of a D2-symmetric tetramer form of KWR**C**IWD which reconciles the intraindole Hδ1−Hζ3 NOE observed. These are interdimer NOEs between equivalent indoles stacking at the interface. This tetramer, with disulfides linking sheets face-to-face rather than edge-to-edge, results in efficient packing of the mostly unchanged dimer units. Conformational exchange between this structure and the C2-symmetric dimer (with the typical edge-to-edge cross-strand disulfides) should be very slow at low temperatures, and this is indeed what we observe by NMR. Specifically, two roughly equal sets of peaks (likely corresponding to dimer and tetramer) are observed at concentrations >1 mM, for this peptide and others of the form $(XWXCXWX)_n$. See Supporting Information for CD and NMR data and a proposed mechanism of exchange.

We observed one another case where evidence existed for a disulfide-swapped (face-to-face instead of edge-to-edge) oligomer. We initially noted a discrepancy between CD and NMR data for the oxidized form of peptide C8 $(RWITKSI\mathbf{C}IRKWE)_n$. The CD (at 30 μM) displayed a weak EtF (edge-to-face) Trp/Trp exciton couplet which decreased rapidly on warming, with an apparent melting point below the coldest assayed temperature (T_M < 280 K), while NMR (measured at 1.5 mM) revealed a near-fully folded species at 280 K. In addition, the NMR spectrum showed a regular CSD pattern suggestive of a canonical β-sheet with none of the serious twisting, buckling, or register shifting expected for a β-sheet with a strained, "backwards diagonal" disulfide bond.

Rerunning the CD experiment in a 0.1 mm CD cell using a portion of the original NMR sample yielded a CD spectra consistent with a high 280 K fold population (χ_F = 0.95 by NMR) and more closely resembling the other peptides of the series (T_M ca. 29 °C; see Figure 4). Clearly an oligomerization is involved, but a specific structural proposal would be purely speculative at this point.

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DISCUSSION

We have provided guidelines for designing stable, disulfide-central β-sheet homodimers and illustrated their structural superiority to the best available hairpin models. Heterodimers can be assembled using the same methods, which expands the potential applications of this strategy. Heterodimer synthesis requires the production of two discrete chains and possesses an innate yield penalty of up to 50%, but if the strands are designed to disfavor homodimerization, yields can approach 100% (as per the greatly favored C6/C8 heterodimer in Figure 3). Two-disulfide ···**CXC**··· sequences oxidize faster and produce β-sheet dimers which are more stable (if also more distorted) compared to their single-disulfide analogues. The limited orthogonality between ···**CXC**··· and ···**C**··· allows for purification of partially oxidized products which could be labeled at the remaining free thiols.

Potential Applications for Disulfide Nucleated β-Sheets. On oxidation, peptide RWKCKCKCKWE formed a single product: the expected antiparallel β-sheet. In addition to providing an example of a highly disulfide-distorted β-sheet (Figures 5 and 8), this rigid, amphipathic, highly cationic dimer

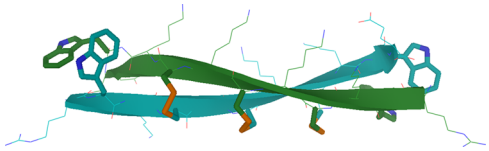

Figure 8. Structure of the "lysine carpet" disulfide dimer $(RWKCKCKCKWE)_2$, with the key disulfide and capping Trp residues highlighted. This structure is supported by NMR data (see Table S10, Supporting Information, for a table of NOEs), but not explicitly generated via annealing to NOE distance constraints.

proved to be a reasonably effective antimicrobial agent with an IC_{50} of 31 μM vs *E. coli* (the details regarding this antibacterial and others will be presented elsewhere).

Disulfide-rich peptides of this form could be used to make a carpet of any polar residue, e.g., a surface made entirely of histidine or aspartic acid sidechains, which have very strong cross-strand His^+/His^+ or Asp^-/Asp^- repulsion. A poly disulfide sheet is the best way to force repulsive residues into a "β-carpet" at pH values where they are entirely charged. In addition to obvious antimicrobial (membrane disruptive) applications (as per the related but synthetically problematic θ-defensin[13,14]), this class of amphipathic peptides could be useful for catalysis,[41] metal binding, or selective detergents[42] and already resembles a common hydrogel-forming structure consisting of alternating valine/lysine.[43−48]

Structurally rigid β-sheets with central disulfides have a range of other potential applications, such as minimalist scaffolds for pharmacophore display, rigid spacers, and building blocks for more complex structures. To further establish the precise structures of disulfide-bound homodimers and thus better apply them to these and other problems, we seek to establish crystal structures for one or more such constructs. While NMR can confirm β-sheet structure and register, it is not always capable of establishing the twist of the hairpin or the nature of any higher-order assembly that takes place (e.g., the precise conformation of an oligomer).

Folding of Disulfide-Mediated β-Sheet Dimers. It is reasonable to assume that disulfide dimer β-sheets fold via a cooperative mechanism, analogous to that of three-stranded sheets[39,40] (see Figure 9.) Once one portion of the β-sheet has formed, a zippering or templating mechanism completes the

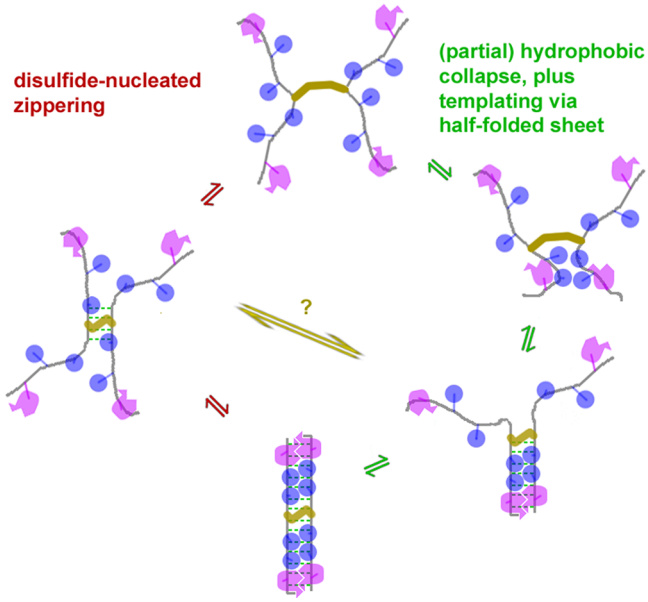

Figure 9. Potential folding mechanisms for disulfide-mediated homodimers. These are analogous to related β-hairpin folding mechanisms of turn nucleation plus zippering and hydrophobic collapse followed by packing optimization and H-bond formation. These folding mechanisms are not mutually exclusive, and even mixed mechanisms (e.g., via the yellow middle path) may be possible.

other half. Theoretically, the folding rate in the forward direction should be faster than that of an equivalent monomeric hairpin, since two degenerate fold pathways exist. The unfolding rate may be slower as well, since a partially unfolded sheet is poised to "zip up" and refold. β-Hairpins can be thought of as "internally cooperative" in the sense that a folded region (either the turn or a sequence-remote strand pair) can promote β-sheet folding in neighboring regions.[49] Thus, turnless β-sheets with disulfides at their centers could be considered "cooperative" (folding of one section catalyzes the folding of another) despite the fact that they are comprised of a single contiguous secondary structure unit. As the inherent fold pathway degeneracy makes it difficult to experimentally distinguish between potential folding mechanisms, molecular dynamics simulations of folding will be essential in analyzing these assumptions.

CONCLUSION

The ease of designing very stable disulfide-nucleated β-sheets suggests that the folding mechanisms of disulfide-rich proteins deserve a closer look. Once thought to be unfavorable,[1−3] we have firmly established that disulfides at cross-strand NHB positions in antiparallel β-sheets are not only favorable (as has since been determined, e.g., refs 4−6) but also more stabilizing for β-folds on a per-chain basis than the best reversing turns. Separate from their role as covalent staples, disulfide bonds have some propensity to form nascent β-sheets, to the benefit (via fold nucleation) or detriment (via kinetic trapping from off-path structures) of a protein's native fold.

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■ ASSOCIATED CONTENT

Supporting Information

CD and NMR data (as chemical shifts, chemical shift deviations, and NOEs), NOE-validated structures of $(RWITKXXXIRKWE)_n$ peptides, a table of the ratios of cystine H_N: neighboring H_N chemical shift deviations, schematic of the folding pathways available to long-loop hairpins dimerized by a cystine in the middle of a long flexible loop. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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