JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

Parallel β-Sheet Secondary Structure Is Stabilized and Terminated by Interstrand Disulfide Cross-Linking

Aaron M. Almeida, Rebecca Li, and Samuel H. Gellman*

Department of Chemistry, University of Wisconsin—Madison, 1101 University Avenue, Madison, Wisconsin 53706, United States

Supporting Information

ABSTRACT: Disulfide bonds between Cys residues in adjacent strands of parallel β-sheets are rare among proteins, which suggests that parallel β-sheet structure is not stabilized by such disulfide cross-links. We report experimental results that show, surprisingly, that an interstrand disulfide bond can stabilize parallel β-sheets formed by an autonomously folding peptide in aqueous solution. NMR analysis reveals that parallel β-sheet structure is terminated beyond the disulfide bond, which causes deviation from the extended backbone conformation at one of the Cys residues.

Helices and sheets are dominant structural motifs within proteins, but these secondary structures are generally not very stable in isolation, particularly for linear peptides of ≤20 residues. Helical conformations (α, 3_{10}, and π) and β-sheets can be stabilized via cross-linking, i.e., macrocycle formation, involving side chains and/or the backbone.[1,2] This structural fortification strategy is observed among biological proteins and peptides, with cyclization most commonly achieved via disulfide formation between cysteine side chains.[3] Other cross-linking modes are found among natural polypeptides as well, and an even wider variety has been explored in synthetic systems.[4] Polypeptides generated via ribosomal biosynthesis and not modified post-translationally, however, are limited to disulfide cross-links, which can form spontaneously under mildly oxidizing conditions.

Bioinformatics analysis of antiparallel β-sheets reveals that pairs of disulfide-linked Cys residues often occur at non-hydrogen-bonded positions that are aligned on adjacent strands[5] (Figure 1). This observation and geometric considerations suggest that such interstrand cross-links can stabilize antiparallel β-sheet secondary structure, a hypothesis that has been supported by numerous studies with designed β-hairpins that fold in aqueous solution.[6] In contrast, disulfide cross-links between adjacent strands are very rare within parallel β-sheets,[5f−h,7] which suggests that disulfide-based macrocyclization should destabilize autonomously folding parallel β-sheets. We report the first test of this hypothesis. Our experiments led to the surprising discovery that an interstrand disulfide can *stabilize* parallel β-sheet secondary structure to one side along the strand direction; however, the parallel sheet cannot propagate beyond the disulfide position.

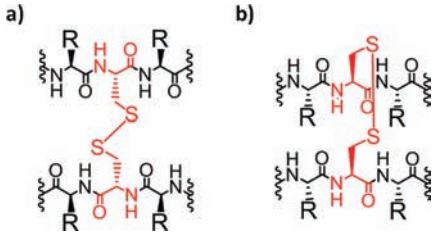

Figure 1. Disulfide bonds between Cys in adjacent strands of (a) antiparallel β-sheet, in non-hydrogen-bonded positions, and (b) parallel β-sheet.

Our experimental design builds upon guidelines we have previously developed for creating short peptides that can form two-stranded parallel β-sheets in aqueous solution.[8] Use of an autonomously folding β-sheet rather than a full-fledged protein is intended to avoid the influence of a specific tertiary context; therefore, our findings should reflect the intrinsic conformational behavior of parallel β-sheet secondary structure. The diamine segment D-prolyl-1,1-dimethyl-1,2-diaminoethane (D-Pro-DADME) promotes but does not enforce β-sheet interactions between peptide segments linked in parallel. Peptide **1-SH** (Figure 2) is related to a molecule previously used to assess the thermodynamics of parallel β-sheet formation in aqueous solution.[8c] The two Cys residues, near the N-terminus of each strand, enable oxidative cyclization to generate **1-SS**. 2D NMR analysis reveals multiple NOEs between protons on residues that are not adjacent in sequence for both forms of **1**, and in each case all NOEs are consistent with the expected parallel β-sheet folding pattern.[9] NOE-restrained molecular dynamics calculations for **1-SS** (based only on NOEs and the CNS force field; no coupling-constant or H-bond constraints were used) suggest canonical parallel β-sheet interactions between the segments bounded by the D-Pro-DADME unit and the Cys residues, i.e., between DFIQV on

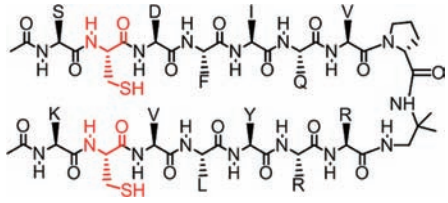

Figure 2. Chemical structure of peptide **1-SH**. Cysteine residues are shown in red.

Received: September 20, 2011
Published: December 8, 2011

   dx.doi.org/10.1021/ja208856c | J. Am. Chem.Soc. 2012, 134, 75−78

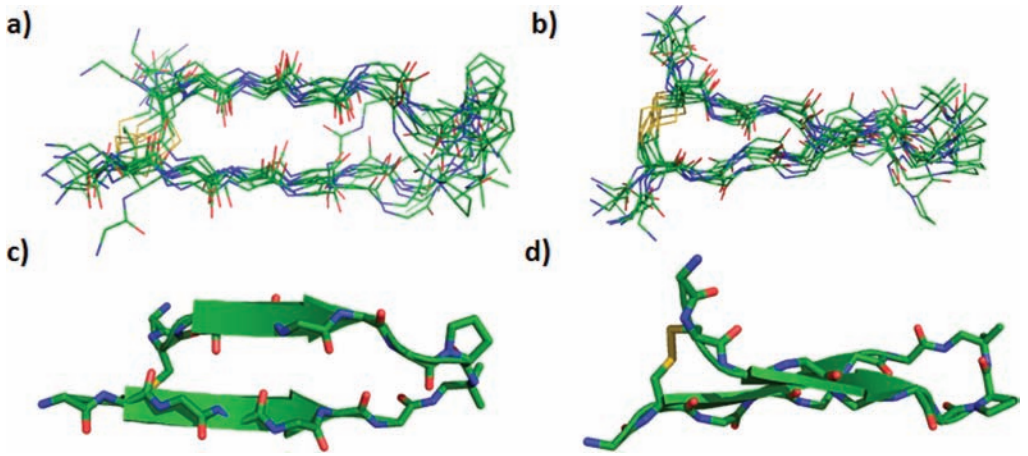

Figure 3. NMR-based structural analysis of **1-SS** based on data obtained for 2.5 mM peptide in 100 mM deuterioacetate, pH 3.8, 4 °C. (a,b) Two views of the overlay of the 10 structures with lowest calculated energies from NOE-restrained molecular dynamics simulations. (c,d) Two views of the average minimized structure; rmsd among backbone atoms is 1.1 ± 0.4 Å.

the upper strand (as drawn) and VLYRR on the lower strand (Figure 3). The β-sheet structure appears to be present throughout the hairpin, up to the location of the disulfide. Only the Cys residue of the lower strand, however, is part of the β-sheet; a kink is observed at the other Cys, which indicates that β-sheet secondary structure is terminated at this point.

Fraying at the open ends of autonomously folding hairpins has previously been noted,[8a,10] but the NMR data suggest that the deviation from local β-sheet structure we observe at the disulfide position in **1-SS** is not attributable to fraying. Comparisons among the 10 lowest-energy structures from the NOE-restrained dynamics calculations for **1-SS** indicate an overall rmsd of 1.1 ± 0.4 Å. Similar rmsd values are obtained for comparisons focused on just a single Cys residue in either strand (1.0 ± 0.3 and 1.1 ± 0.5 Å, respectively, for the top and bottom Cys residues as drawn in Figure 2). If these Cys residues were frayed relative to the core of the β-sheet, then the Cys rmsd values should be larger than the overall rmsd.

For a residue that participates in β-sheet secondary structure, the α-proton chemical shift ($\delta C_\alpha H$) is generally downfield of the position expected for that residue in an unfolded ("random coil") state.[11] We have shown that $\delta C_\alpha H$ data can be used to assess the parallel β-sheet population for molecules such as **1-SH**,[8c−e] which are anticipated to equilibrate rapidly between folded and unfolded states on the NMR time scale. This analysis requires two reference compounds, one to provide $\delta C_\alpha H$ values for the fully unfolded state and another to provide $\delta C_\alpha H$ values for the fully folded state. The former goal is achieved by replacing D-Pro in the diamine linker with L-Pro,[8a] and the latter goal is achieved by backbone cyclization with a diacid linking segment.[8c,d,9]

We used this approach to estimate the extent of parallel β-sheet folding in **1-SH** and **1-SS** by focusing on four "indicator residues", Ile and Gln in the upper strand and Tyr and the more N-terminal Arg in the lower strand (Figure 2). These residues provide four independent measurements of parallel β-sheet population, and each is sufficiently isolated from the variable C-terminal portion of its strand to be free of chemical shift influences induced by covalent changes. For **1-SH**, $\delta C_\alpha H$ values for all four indicator residues are significantly downfield of the $\delta C_\alpha H$ values in the unfolded reference peptide (Figure 4), which is consistent with the NOE data in indicating substantial parallel β-sheet formation. However, the indicator $\delta C_\alpha H$ values

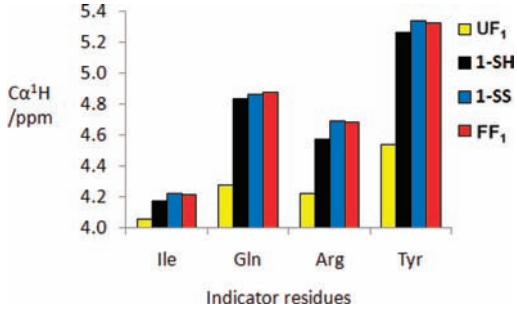

Figure 4. ^{1}H NMR chemical shift data for protons attached to C_α ($\delta C_\alpha H$) of indicator residues in the unfolded reference peptide ($\mathbf{UF_1}$), **1-SH**, **1-SS**, and the folded reference peptide ($\mathbf{FF_1}$).

for **1-SH** do not differ significantly from the corresponding values for **1-SS** or the fully folded reference peptide, which makes it impossible to determine whether interstrand disulfide formation enhances parallel β-sheet stability.

We examined a second design that was intended to manifest a lower folding propensity in order to assess the thermodynamic impact of an interstrand disulfide on parallel β-sheet

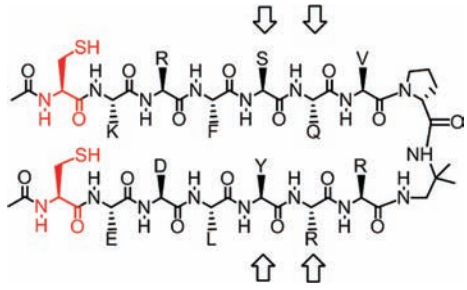

Figure 5. Chemical structure of peptide **2-SH**. Cysteine residues are shown in red. Indicator residues are denoted by arrows.

stability. The sequence of **2-SH**/**2-SS** (Figure 5) differs at several points from that of **1-SH**/**1-SS**. Two changes are particularly noteworthy: (1) the Cys residues occur at the N-termini of the strands in **2**, rather than adjacent to the N-termini in **1**, and (2) a cross-strand Ile-Tyr pairing that was intended to provide a hydrophobic driving force for folding of **1** has been replaced by a Ser-Tyr pairing, which should be less

dx.doi.org/10.1021/ja208856c | J. Am. Chem.Soc. 2012, 134, 75−78

conducive to parallel β-sheet formation. 2D NMR analysis suggests qualitatively that **2-SH** and **2-SS** display smaller extents of parallel β-sheet folding relative to **1-SH** and **1-SS**, because the number of interstrand NOEs is smaller for both forms of **2** than for either form of **1**.[9] Nevertheless, the 2D NMR data for **2-SH** and **2-SS** indicate adoption of the expected parallel β-sheet conformation, in the population of molecules that are folded, because all NOEs involving protons from sequentially nonadjacent protons are consistent with this conformation.[9]

The extent of parallel β-sheet folding for **2-SH** and **2-SS** in aqueous solution was estimated on the basis of $\delta C_\alpha H$ data, via comparisons with appropriate fully folded and fully unfolded reference peptides.[8e,9] Following the approach used for series **1**, we focused on $\delta C_\alpha H$ data from four indicator residues, Ser and Gln in the upper strand and Tyr and the more N-terminal Arg in the lower strand. At each position, $\delta C_\alpha H$ values show a steady downfield movement in the order unfolded reference, **2-SH**, **2-SS**, folded reference (Figure 6a). This consistent trend qualitatively suggests that neither version of **2** is fully folded and, more important, that disulfide formation causes an increase in the extent of parallel β-sheet formation. $\delta C_\alpha H$-based population analysis[9] provides a reasonably consistent conclusion across the four indicator positions, suggesting that **2-SH** is ~20% folded and that **2-SS** is ~70% folded in 9:1 H_2O:D_2O, pH 3.8, 100 mM sodium deuterioacetate buffer (buffer pH was not corrected for isotope effects) at 4 °C (Figure 6b). These folded state population values can be converted to Gibbs free energy of folding (ΔG_F) on the basis of a two-state conformational model, random coil vs parallel β-sheet conformation.[8c−e] Data from the four indicator residues suggest a $\Delta\Delta G_F$ value of −1.1 ± 0.1 kcal/mol, which shows that the interstrand disulfide in **2-SS** provides significant stabilization to the parallel β-sheet conformation.

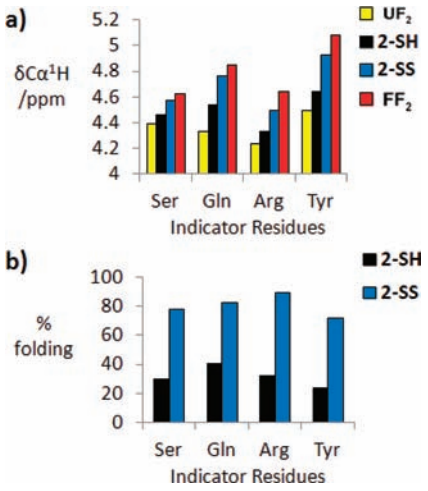

Figure 6. (a) ^{1}H NMR chemical shift data for protons attached to C_α ($\delta C_\alpha H$) of indicator residues in the unfolded reference peptide ($\mathbf{UF_2}$), **2-SH**, **2-SS**, and the folded reference peptide ($\mathbf{FF_2}$). (b) Percent folding calculated for **2-SH** and **2-SS** on the basis of data obtained for 2.5 mM peptide in 100 mM deuterioacetate, pH 3.8, 4 °C.

Previous bioinformatics analysis suggested that interstrand disulfide cross-links are not well accommodated in parallel β-sheet secondary structure,[5f−h,7] and this conclusion is supported by our experimental findings with autonomously folding peptides, since the backbone kinks at the disulfide. However, our studies reveal an unexpected insight: an interstrand disulfide can stabilize parallel β-sheet secondary structure that forms in the C-terminal direction relative to the Cys residues. Covalent bonding between Cys side chains appears to require that the backbone deviate from the extended conformation, at least in one strand, which prevents the sheet from propagating through the disulfide position in our peptides. Overall these results suggest that interstrand cystine cross-links can both stabilize and define the extent of parallel β-sheet secondary structure in designed peptides and proteins.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details including compound characterization, NMR data, and thermodynamic analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

gellman@chem.wisc.edu

■ ACKNOWLEDGMENTS

This research was supported by the NIH (GM061238). R.L. was supported in part by fellowship from the UW Department of Chemistry and by NSF grant CHE-0848847. NMR spectrometers were purchased with partial support from NIH (Grant No. 1 S10 RR13866-01) and NSF.

■ REFERENCES

(1) (a) Henchey, L. K.; Jochim, A. L.; Arora, P. S. *J. Am. Chem. Soc.* **2008**, *12*, 692−697. (b) Phelan, J. C.; Skelton, N. J.; Braisted, A. C.; McDowell, R. S. *J. Am. Chem. Soc.* **1997**, *119*, 455−460. (c) Haubner, R.; Schmitt, W.; Holzemann, G.; Goodman, S. L.; Jonczyk, A.; Kessler, H. *J. Am. Chem. Soc.* **1996**, *118*, 7881−7891. (d) Lutgring, R.; Chmielewski, J. *J. Am. Chem. Soc.* **1994**, *116*, 6451−6452. (e) Betz, S. F. *Protein Sci.* **1993**, *2*, 1551−1558. (f) Harrison, P. M.; Sternberg, M. J. E. *J. Mol. Biol.* **1994**, *244*, 448−463.

(2) Selected examples: (a) Felix, A. M.; Heimer, E. P.; Wang, C. T.; Lambros, T. J.; Fournier, A.; Mowles, T. F.; Maines, S.; Campbell, R. M.; Wegrzynski, B. B.; Toome, V.; Fry, D.; Madison, V. S. *Int. J. Pept. Protein Res.* **1988**, *32*, 441−454. (b) Osapay, G.; Taylor, J. W. *J. Am. Chem. Soc.* **1992**, *114*, 6966−6973. (c) Leduc, A. M.; Trent, J. O.; Wittlif, J. L.; Bramlett, K. S.; Briggs, S. L.; Chirgadze, N. Y.; Wang, Y.; Burris, T. P.; Spatola, A. F. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 11273−11278. (d) Zhou, H. X. X.; Hull, L. A.; Kallenbach, N. R.; Mayne, L.; Bai, Y. W.; Englander, S. W. *J. Am. Chem. Soc.* **1994**, *116*, 6482−6483. (e) Jackson, D.; King, D.; Chmielewski, J.; Singh, S.; Schultz, P. *J. Am. Chem. Soc.* **1991**, *113*, 9391−9392.

(3) (a) McDonald, N. Q.; Hendrickson, W. A. *Cell* **1993**, 421−424. (b) Willey, J. M.; van der Donk, W. A. *Annu. Rev. Microbiol.* **2007**, *61*, 477−501. (c) Ganz, T. *Nat. Rev. Immunol.* **2003**, *3*, 710−720. (d) Cheek, S.; Krishna, S. S.; Grishin, N. V. *J. Mol. Biol.* **2006**, *359*, 215−237. (e) Lin, S. L.; Nussinov, R. *Nat. Struct. Biol.* **1995**, *2*, 835−837. (f) Gause, G. F.; Brazhnikova, M. G *Nature* **1944**, *154*, 703.

(4) Selected examples: (a) Ghadiri, M. R.; Choi, C. *J. Am. Chem. Soc.* **1990**, *112*, 1630−1632. (b) Ruan, F. Q.; Chen, Y. Q.; Hopkins, P. B. *J. Am. Chem. Soc.* **1990**, *112*, 9403−9404. (c) Tatko, C. D.; Waters, M. L. *J. Am. Chem. Soc.* **2002**, *124*, 9372−9373. (d) Kelso, M. J.; Beyer, R. L.; Hoangt, H. N.; Lakdawala, A. S.; Snyder, J. P.; Oliver, W. V.; Robertson, T. A.; Appleton, T. G.; Fairlie, D. P. *J. Am. Chem. Soc.* **2004**, *126*, 4828−4842. (e) Blackwell, H. E.; Grubbs, R. H. *Angew. Chem., Int. Ed.* **1998**, *37*, 3281−3284. (f) Bernal, F.; Tyler, A. F.; Korsmeyer, S. J.; Walensky, L. D.; Verdine, G. L. *J. Am. Chem. Soc.* **2007**, *129*, 2456−2457. (g) Walensky, L. D.; Kung, A. L.; Escher, I.; Malia, T. J.; Barbuto, S.; Wright, R. D.; Wagner, G.; Verdine, G. L.; Korsmeyer, S. J. *Science* **2004**, *305*, 1466−1470. (h) Nowick, J. S. *Acc.*

Chem. Res. **2008**, *41*, 1319−1330. (i) Fujimoto, K.; Kajino, M.; Inouye, M. *Chem.—Eur. J.* **2008**, *14*, 857−863. (j) Liu, J.; Wang, D.; Zheng, Q.; Lu, M.; Arora, P. S. *J. Am. Chem. Soc.* **2008**, *130*, 4334−4337. (k) Wang, D.; Chen, K.; Kulp, J. L. III; Arora, P. S. *J. Am. Chem. Soc.* **2006**, *128*, 9248−9256. (l) Haney, C. M.; Loch, M. T.; Horne, W. S. *Chem. Commun.* **2011**, *47*, 10915−10917. (m) Davies, J. S. *J. Pept. Sci.* **2003**, *9*, 471−501. (n) Miller, S. J.; Blackwell, H. E.; Grubbs, R. H. *J. Am. Chem. Soc.* **1996**, *118*, 9606−9614. (o) Schafmeister, C. E.; Po, J.; Verdine, G. L. *J. Am. Chem. Soc.* **2000**, *122*, 5891−5892. (p) Brunel, F. M.; Dawson, P. E. *Chem. Commun.* **2005**, 2552−2554. (q) Judice, J. K.; Tom, J. Y.; Huang, W.; Wrin, T.; Vennari, J.; Petropoulos, C. J.; McDowell, R. S. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 13426−13430. (r) Sia, S. K.; Carr, P. A.; Cochran, A. G.; Malashkevich, V. N.; Kim, P. S. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 14664−14669. (s) Balaram, H.; Uma, K.; Balaram, P. *Int. J. Pept. Protein Res.* **1990**, *35*, 495−500. (t) Holland-Nell, K.; Meldal, M. *Angew. Chem.* **2011**, *123*, 5310−5312. (u) Liu, C.; Sawaya, M. R.; Cheng, P. N.; Zheng, J.; Nowick, J. S.; Eisenberg, D. *J. Am. Chem. Soc.* **2011**, *133*, 6736−6744.

(5) (a) Hutchinson, E. G.; Sessions, R. B.; Thornton, J. M.; Woolfson, D. N. *Protein Sci.* **1998**, *7*, 2287−2300. (b) Wouters, M. A.; Curmi, P. M. G. *Proteins: Struct. Funct. Genet.* **1995**, *22*, 119−131. (c) Lifson, S.; Sander, C. *J. Mol. Biol.* **1980**, *139*, 627−639. (d) Richardson, J. S. *Adv. Protein Chem* **1981**, *34*, 167−339. (e) Harrison, P. M.; Sternberg, M. J. *J. Mol. Biol.* **1996**, *264*, 603−623. (f) Srinivisan, N.; Sowdhamini, R.; Ramakrishnan, C.; Balaram, P. *Int. J. Pept. Protein Res.* **1990**, *36*, 147−155. (g) Mao, B. *J. Am. Chem. Soc.* **1988**, *111*, 6132−6136. (h) Chou, P. Y.; Fasman, G. D. *Biochemistry* **1974**, *13*, 221−222. (i) Levitt, M. *Biochemistry* **1978**, *17*, 4277−4285.

(6) Selected examples: (a) Cochran, A. G.; Tong, R. T.; Starovasnik, M. A.; Park, E. J.; McDowell, R. S.; Theaker, J. E.; Skelton, N. J. *J. Am. Chem. Soc.* **2001**, *123*, 625−632. (b) Gunasekaran, K.; Ramakrishnan, C.; Balaram, P. *Protein Eng.* **1997**, *10*, 1131−1141. (c) Santiveri, C. M.; Leon, E.; Rico, M.; Jimenez, M. A. *Chemistry* **2008**, *14*, 488−499. (d) Martinek, T. A.; Fulop, F. *Eur. J. Biochem.* **2003**, *270*, 3657−3666. (e) Mirassou, Y.; Santiveri, C. M.; Perez de Vega, M. J.; Gonzalez-Muniz, R.; Jiminez, M. A. *ChemBioChem* **2009**, *10*, 902−910.

(7) Fooks, H. M.; Martin, A. C.; Woolfson, D. N.; Sessions, R. B.; Hutchinson, E. G. *J. Mol. Biol.* **2006**, *356*, 32−44.

(8) (a) Fisk, J. D.; Gellman, S. H. *J. Am. Chem. Soc.* **2001**, *123*, 343−344. (b) Fisk, J. D.; Powell, D. R.; Gellman, S. H. *J. Am. Chem. Soc.* **2000**, *122*, 5443−5447. (c) Fisk, J. D.; Schmitt, M. A.; Gellman, S. H. *J. Am. Chem. Soc.* **2006**, *128*, 7148−7149. (d) Freire, F.; Gellman, S. H. *J. Am. Chem. Soc.* **2009**, *131*, 7970−7972. (e) Freire, F.; Almeida, A. M.; Fisk, J. D.; Steinkruger, J. D.; Gellman, S. H. *Angew. Chem.* **2011**, *50*, 8735−8738.

(9) See the Supporting Information for details.

(10) Selected examples: (a) Baldwin, R. L. *Biophys. Chem.* **1995**, *55*, 127−135. (b) de Alba, E.; Jimenez, M. A.; Rico, M. *J. Am. Chem. Soc.* **1997**, *119*, 175−183. (c) Syud, F. A.; Stanger, H. E.; Gellman, S. H. *J. Am. Chem. Soc.* **2001**, *123*, 8667−8677. (d) Ciani, B.; Jourdan, M.; Searle, M. S. *J. Am. Chem. Soc.* **2003**, *125*, 9038−9047. (e) Rai, R.; Raghothama, S.; Balaram, P. *J. Am. Chem. Soc.* **2006**, *128*, 2675−2681. (f) Rieman, A. L.; Waters, M. L. *J. Am. Chem. Soc.* **2009**, *131*, 14081−14087. (g) Kier, B. L.; Shu, I.; Eidenschink, L. A.; Andersen, N. H. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 10466−10471. (h) Eidenschink, L.; Crabbe, E.; Andersen, N. H. *Biopolymers* **2009**, *91*, 557−564.

(11) Wishart, D. S.; Sykes, B. D.; Richards, F. M. *Biochemistry* **1992**, *31*, 1647−1651.