Design and Construction of an Open Multistranded β-Sheet Polypeptide Stabilized by a Disulfide Bridge

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Received October 30, 2001

Abstract: The design and characterization of an open eight-stranded β-sheet in a synthetic, 2-fold symmetric 70-residue peptide is described. The design strategy involves the generation of a 35-residue four-stranded β-sheet peptide in which successive hairpins are nucleated by appropriately positioned DPro—Xxx sequences. Oxidative dimerization using a single Cys residue positioned at the center of the C-terminal strand results in a disulfide-bridged eight-stranded structure. Nuclear Overhauser effects firmly establish an eight-stranded β-sheet in methanol. In water, the outer strands are frayed, but a well-defined four-stranded β-sheet stabilized by a disulfide bridge and a hydrophobic cluster is determined from NMR data. Comparison of the precursor peptide with the disulfide-bridged dimer reveals considerable enhancement of β-sheet content in the latter, suggesting that the disulfide cross-link is an effective strategy for the stabilization of β-sheets.

Introduction

β-sheets in proteins are generally stabilized by packing into hydrophobic cores, facilitating solvent exclusion and consequent enhancement of the energetic contributions due to cooperative interstrand hydrogen bonding. Large amphipathic β-sheets can curve to form closed barrels, as exemplified by the structures of the transmembrane porins.[1] Single-layer β-sheets without a hydrophobic core and with the central three strands completely exposed to bulk solvent have been characterized in the outer surface protein A (OspA) of *Borrelia burgdorferi*.[2] Using the OspA sequence as a starting point, larger single-layer β-sheets were designed.[3] We report here the first principles design and construction of an open eight-stranded β-sheet, in a synthetic 2-fold symmetric 70-residue peptide, employing a strategically placed disulfide bridge for stabilizing the core of the β-sheet.

Our approach to β-sheet construction is based on the ability of DPro—Xxx sequences to nucleate β-turns of appropriate stereochemistry ("prime" turns).[4,5] Alternative turn-forming strategies have used Asn—Gly segments.[4b,6] A comparison of the relative abilities of the two turn-nucleating segments has established DPro—Xxx sequences as superior hairpin nucleators.[7] The positioning of multiple turn-forming nuclei within a designed sequence permits the construction of multistranded β-sheets. Thus far, three- and four-stranded β-sheets so designed have exhibited considerable stability in nonaqueous[8] and mixed aqueous solvents,[9] although they have been fragile in aqueous media. However, a large number of three-stranded β-sheets have been constructed that fold in water,[10] and redesign of a portion of the toxin hand fold resulted in a three-stranded "mini-protein" that contains a compact hydrophobic core.[11]

The de novo design and structural characterization of larger domains that contain predominantly extended structure is a more

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(5) Abbreviations: All amino acids are of the L-configuration unless otherwise mentioned. DPro, D-proline; NOE, nuclear Overhauser effect; Acm, acetamidomethyl; Trt, triphenylmethyl; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser enhancement and exchange spectroscopy; DQF-COSY, double quantum filtered correlation spectroscopy; ESI-MS, electrospray ionization mass spectrometry; ANS, 8-anilino-1-naphthalenesulfonic acid
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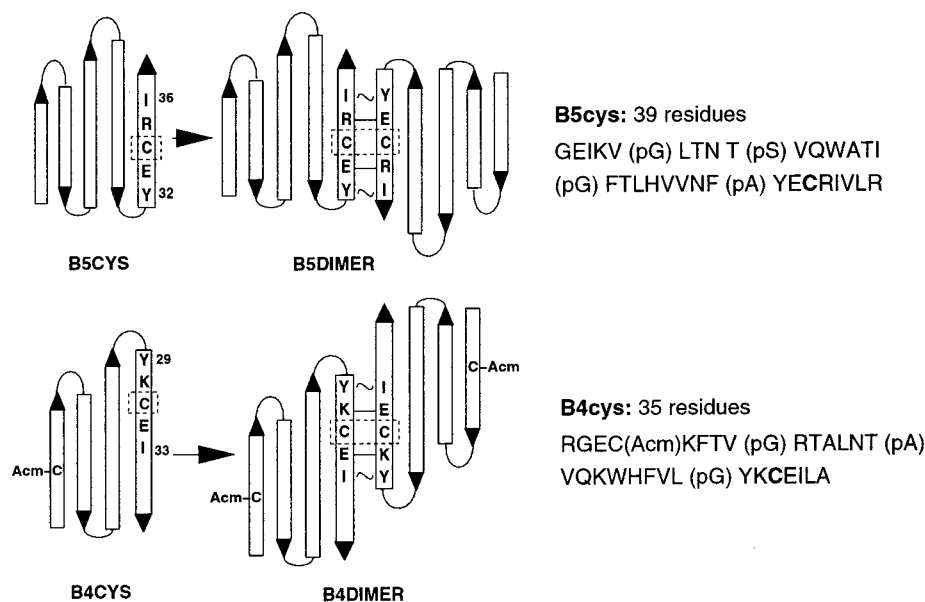(11) Ottesen, J. J.; Imperiali, B. *Nat. Struct. Biol.* **2001**, *8*, 535.

B5cys: 39 residues

GEIKV (pG) LTN T (pS) VQWATI
(pG) FTLHVVNF (pA) YECRIVLR

B4cys: 35 residues

RGEC(Acm)KFTV (pG) RTALNT (pA)
VQKWHFVL (pG) YKCEILA

Figure 1. Schematic representation of the design of the β-sheet peptides. **B5**cys and **B4**cys, the precursor peptides, bear Cys residues in the middle of the terminal strand. Oxidative dimerization results in the disulfide-bonded peptide, **B5**dimer and **B4**dimer, respectively. Key: p, ^{D}Pro residue; residues in parentheses, potential turn forming segments; −, potential hydrogen bond; ∼, interacting residue pairs generated on disulfide bond formation.

formidable task, as the isolated β-sheet in solution has a pronounced tendency to aggregate laterally to form extended hydrogen-bonded networks. The resultant complexes are large, heterogeneous, and structurally intractable, accounting for the many difficulties associated with the construction of discrete β-sheet units in solution.[12] In attempts to construct β-sandwich modules, Mayo and co-workers modified the β-sheet domain of the α-chemokines to design a tetrameric three-stranded β-sheet in which the three-stranded β-sheet units assemble into C_2 symmetric six-stranded sheets.[13]

This study has two major goals: to construct large, extended β-sheets in solution that adopt primarily open structures and to explore the efficacy of the disulfide bridge as a device for the stabilization of such structures. Disulfide bridges can be stereochemically accommodated in the non-hydrogen-bonding positions of antiparallel β-sheets.[14] Our approach envisaged a strategy wherein edge strands of multiply stranded β-sheets are covalently linked through disulfide bonds to form extended β-sheets. The construction of large β-sheet structures could, in principle, be achieved by the judicial placement of turn-forming ^{D}Pro−Xxx sequences within a single polypeptide chain. However, the chemical synthesis of polypeptide chains by the sequential assembly of residues on a solid support is still a formidable task for chain lengths of over, say, 50 residues. This

necessitates the synthesis of smaller fragments (which form multiple-stranded β-sheets) and their subsequent assembly using disulfide bonds.[15]

Results and Discussion

Design of the β-Sheet Motifs. Initial attempts involved the design of a 39-residue, five-stranded β-sheet, **B5**cys, with a Cys residue placed at the center of the C-terminal strand (C34), such that oxidation to form a disulfide bridge would yield a C_2 symmetric 10-stranded β-sheet structure, **B5**dimer (Figure 1).

The sequence in the strands contains several β-branched residues (Thr/Ile/Val), which have high propensity to occur in β-sheets. Charged residues were also placed in the N- and C-terminal strands in order to discourage lateral aggregation.[6c] The sequence contained single Tyr/Trp residues intended to act as spectroscopic reporters. Arginine (R35) and glutamic acid (E33) residues were placed flanking Cys(34) to result in stabilizing electrostatic interactions across the β-strands forming the dimer interface. A unique tyrosine residue was positioned such that, observation of aromatic methyl NOEs between Y32 and I36 would confirm the presence of β-sheet structure, with the desired strand registry across the dimer interface (Figure 1). It should be noted that no intrasubunit aromatic/methyl NOE involving the Tyr residue is possible in the designed peptide.

The circular dichroism spectra of **B5**cys in methanol yielded a negative CD band at 216 nm, which is characteristic of β-sheet structure.[16] Oxidative dimerization of **B5**cys formed the disulfide-bonded peptide, **B5**dimer, which exhibited a more intense negative CD band at 215 nm (*mean residue* ellipticity values: **B5**cys −6.81 × 10^{-3} deg·cm^2·dmol^{-1}; **B5**dimer −14.3 × 10^{-3}

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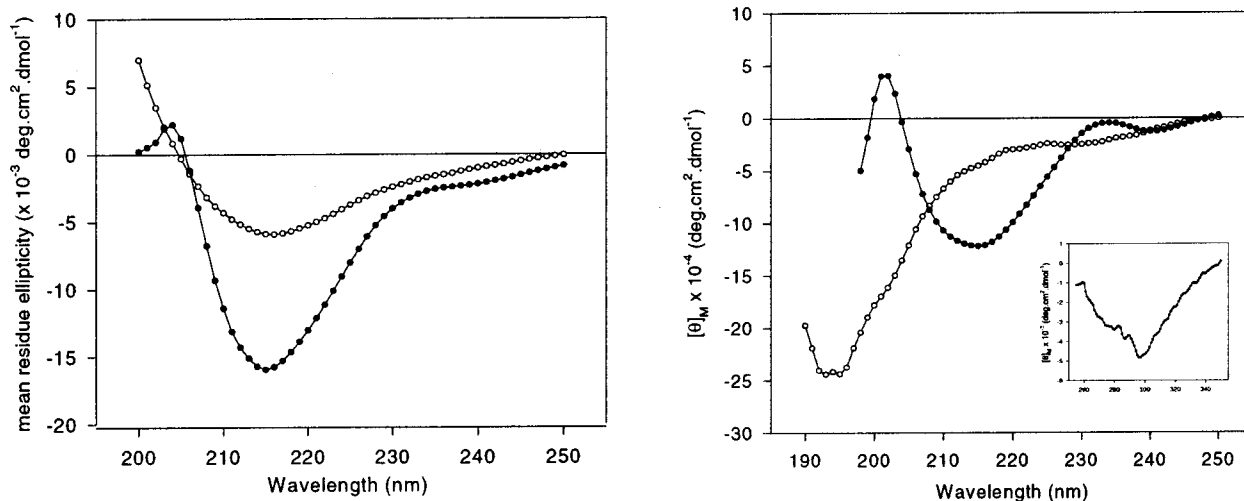
Figure 2. CD spectra of **B4**cys (○) and **B4**dimer (●) in methanol (left) and water (right) ([θ]$_M$, molar ellipticity). **B4**dimer also displays near-UV CD (inset), which is rarely observed for synthetic peptides. Peptide concentrations, ~40 μM; path length, 0.1 cm (far-UV), 1 cm (near-UV).

deg·cm²·dmol⁻¹), indicating that disulfide bridging enhances the β-sheet content of the peptide as anticipated. While the precursor peptide **B5**cys was soluble in water and yielded a CD spectrum similar to that in methanol, **B5**dimer was largely insoluble. We therefore turned to an alternate sequence, **B4**cys, which contained many charged residues to enhance solubility. The sequence of the 35-residue **B4**cys peptide, illustrated in Figure 1 (bottom), contained Cys residues positioned at positions 4 and 31, in the center of both N- and C-terminal strands. Differential protection (Acm and Trt) of the two Cys residues was employed to permit selective oxidation of the C31 residue, following simultaneous removal of the Trt group during acid deprotection from the resin, to yield a 70-residue potentially eight-stranded β-sheet peptide (Figure 1). The placement of C4 was designed to permit eventual elaboration to a closed barrel-like 8- or 16-stranded structure. The registry of residues across strands was staggered to be compatible with an eight-stranded ($n = 8$) β-barrel with a shear number of 8 ($S = 8$).[17] This necessitated an increase in strand length, and hence, the strand number was reduced to four, to restrict peptide length. The sequence of **B4**cys retained the many features designed to aid NMR characterization in **B5**cys, such as the appropriate positioning of aromatic/aliphatic and charged residue pairs across the potential dimer interface (Figure 1). The four-stranded template of **B4**cys also contained many aromatic residues, positioned at the center of the β-sheet, to form a hydrophobic cluster that may serve as a nucleation center for folding in water.

Preliminary CD analysis of **B4**cys and **B4**dimer in methanol yielded results similar to those observed in the first-generation molecules (**B5**cys and **B5**dimer): the CD spectra of both molecules resemble β-sheets (Figure 2, left) and are characterized by large values of molar ellipticity. Comparison of the mean residue ellipticity values of both **B4**cys and **B4**dimer (**B4**cys, 216 nm: 5.92×10^{-3} deg·cm²·dmol⁻¹. **B4**dimer, 215 nm: 15.95×10^{-3} deg·cm²·dmol⁻¹) reinforced the earlier observation that the disulfide bond stabilizes β-sheet conformation in the dimeric molecule. Most dramatically, the disulfide-bonded peptide **B4**dimer showed a significantly intense CD band in water at 215 nm, in marked contrast to **B4**cys (Figure 2, right).

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B4dimer even displayed near-UV CD, often considered indicative of tertiary structure, but rarely observed in de novo designed peptides (Figure 2, right, inset).

NMR Spectroscopy of B4dimer in Water and Methanol. The 500-MHz ¹H NMR spectra of the **B4**cys peptide in methanol and water reveal broad lines and poor resolution, features often observed in the spectra of molecules that undergo extensive aggregation in solution. Significantly, the NMR spectra of **B4**dimer in both solvents were well resolved with relatively sharp resonances, suggesting the absence of pronounced peptide aggregation. Indeed, gel filtration experiments in water (data not shown), performed at peptide concentrations used in NMR experiments, indicated the absence of large aggregates for **B4**dimer, although some dimer formation was observed. The observation of large chemical shift dispersion for the C^{α}H (methanol, 3.64–5.89 ppm; water, 3.55–5.63 ppm) and NH (methanol, 8.02–9.49 ppm; water, 7.82–9.01 ppm) protons and the appearance of high-field-shifted methyl resonances (methanol, 0.57, 0.61 ppm; water, 0.64 ppm) suggested the formation of well-defined structures. Complete sequence-specific assignment of residues in both methanol and water was accomplished using a combination of TOCSY, DQF-COSY, and NOESY experiments (chemical shift table given as Supporting Information). Notably, analysis of the TOCSY spectra resulted in the identification of only 35, and not 70, spin systems, establishing the presence of C_2 symmetry in the dimeric molecule. Careful examination of the TOCSY spectra of **B4**dimer does reveal the presence of a few unassigned correlations, which might be due to small populations of minor conformations.

Peptide Conformation in Methanol. (a) The B4 Monomeric Unit. The NOESY spectrum of **B4**dimer in methanol contains intense C$_i^α$H/N$_{i+1}$H and very weak (and at times, absent) C$_i^α$H/N$_i$H NOEs, a feature reminiscent of β-sheet conformations. The presence of β-conformations was reinforced by the fact that the *only* sequential NH/NH NOEs observed were between residues at "$i + 2$" and "$i + 3$" positions of the designed tight turns [Gly(10) ↔ Arg(11), Ala(18) ↔ Val(19), and Gly(28) ↔ Tyr(29)]. This also supported local helical conformations ($α_R/α_L$) at residues Gly(10), Ala(18), and Gly-(28). The ^{D}Pro–Xxx sequence can adopt either type II′ or I′
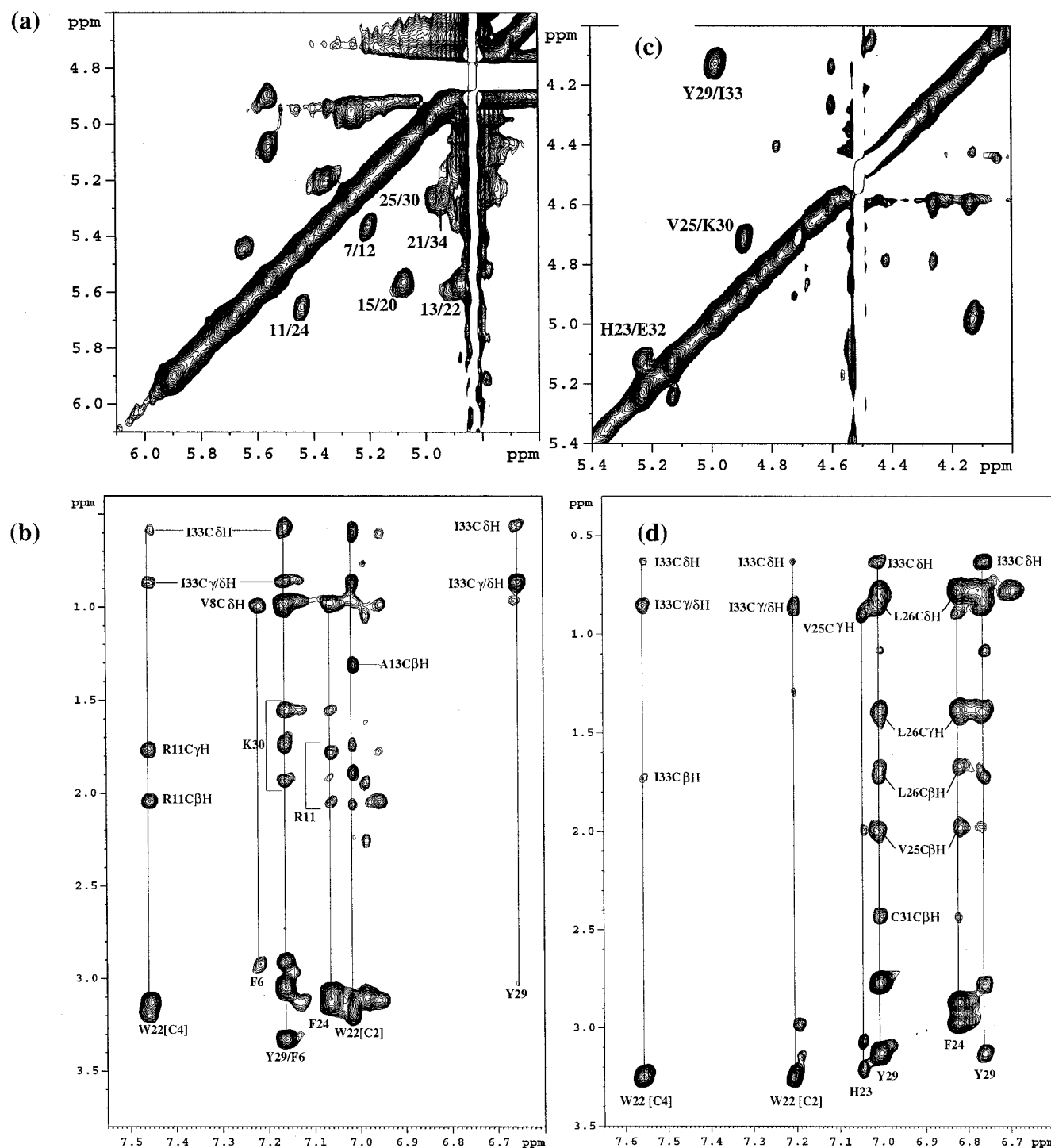
Figure 3. Partial expansion of the NOESY spectra of **B4**dimer (a) in CD$_3$OD (300 K), illustrating cross-strand C$^\alpha$H/C$^\alpha$H NOEs, (b) in CD$_3$OD (300 K) illustrating NOEs between aromatic and aliphatic side chains, and (c) in D$_2$O (323 K), illustrating observed C$^\alpha$H/C$^\alpha$H NOEs. (d) The key side-chain NOEs observed in water (D$_2$O, 323 K).

β-turn conformations. The former would require the Xxx residue to adopt an α_R conformation while the latter requires an α_L conformation.[18] The additional observation of strong N$_i$H/C$_i^\alpha$H ($d_{N\alpha}$) and weak C$_i^\alpha$H/N$_{i+1}$H ($d_{\alpha N}$) NOEs involving these residues and intense $d_{\alpha N}$ (i, $i + 1$) NOEs between ^{D}Pro(8) $\leftrightarrow$

Gly(10), ^{D}Pro(17) $\leftrightarrow$ Ala(18), and ^{D}Pro(27) $\leftrightarrow$ Gly(28) (which correspond to a ψ_{DPro} value close to $-120°$) implies that all three turns adopt the type II$'$ conformation.

Evidence for the formation of a multiple-stranded β-sheet with desired strand registry is obtained by the observation of a number of key cross-strand NH/NH (F6/A13, V8/R11, T12/H23, L14/K21, T16/V19, W22/I33, L26/Y29) and C$^\alpha$H/C$^\alpha$H NOEs (Figure 3a). The F24/C31 NH/NH NOE and K5/L14, H23/E30 C$^\alpha$H/C$^\alpha$H NOEs, which are expected in the experi-

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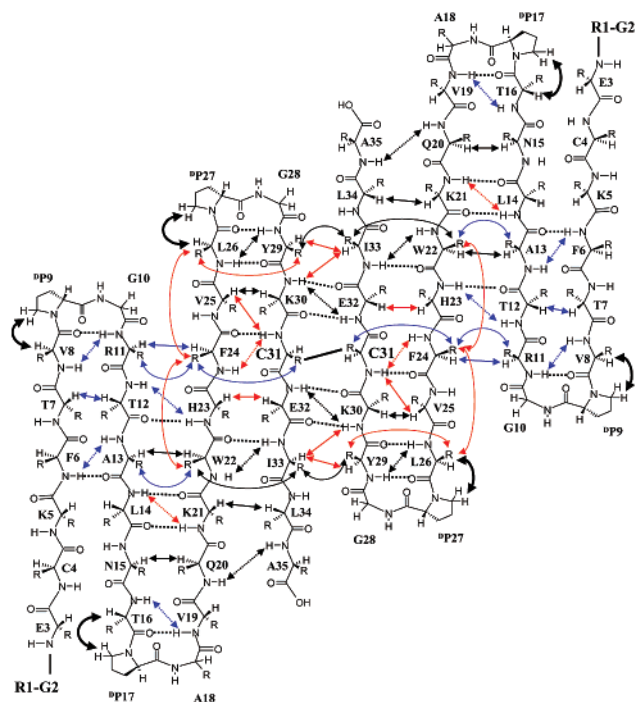
Figure 4. Schematic representation of the backbone of **B4**dimer illustrating long-range NOEs observed in water and in methanol. NOEs observed in both solvents are black, NOEs observed only in water are red, and those seen only in methanol are blue. Unbroken arrows depict medium-intensity NOEs, dotted arrows represent weak NOEs, thick curved arrows depict strong Xxx(CᵅH)−DPro(CᵟH) NOEs, and thin curved arrows indicate interacting side chains (these represent more than one NOE). The disulfide bond is shown as a straight line between the two Cys(31) residues.

mentally obtained β-sheet structure for **B4**dimer, are not observed due to resonance overlap of amide/Cᵅ protons.

A consequence of folded conformations is the spatial proximity of side chains distant in amino acid sequence. This is reflected in the NOESY spectrum of **B4**dimer, which is rich in long-range side-chain−side-chain correlations. Figure 3c delineates NOEs between many aromatic and aliphatic side chains which could be unambiguously assigned. The structure of **B4**dimer is characterized by the presence of a network of aromatic/side-chain interactions. The aromatic ring protons of Trp(22) and Phe(24) give rise to many NOEs with residues in adjacent strands (W22 ↔ A13, W22 ↔ I33, W22 ↔ R11, F24 ↔ R11, F24 ↔ C31), the Cᵝ protons of A13 particularly undergoing a dramatic upfield shift to 0.61 ppm under the influence of the Trp(22) ring. The observation of such interactions suggests the formation of a local hydrophobic cluster, which stabilizes the extended β-sheet of **B4**dimer.

Figure 4 illustrates a summary of the NMR data with observed NOEs marked on the structure of **B4**dimer. The NOEs discussed thus far confirm a four-stranded β-sheet structure in the monomeric **B4** unit.

(b) Interactions across the Dimer Interface. A striking feature of the NOESY spectrum of **B4**dimer is the presence of a medium intensity correlation between the amide protons of residues Lys(30) and Glu(32) (Figure 4), confirming the antiparallel alignment of the disulfide-bonded interface strands of the dimeric β-sheet. Unfortunately, it was not possible to precisely identify the expected CᵅH/CᵅH NOE between residues Tyr(29) and Ile(33) in the NOESY spectrum of **B4**dimer recorded after complete exchange of labile protons in CD₃OD,

as the chemical shift of the Cᵅ proton of Ile(33) occurs too close to the water signal. But, significantly, NOEs between the methyl groups of Ile(33) and the aromatic ring of Tyr(29) were observed; both residues are present at opposite ends of strand 4 in the monomeric unit of **B4**dimer. This is consistent with cross-strand side-chain interactions at the dimer interface, where Ile(33) and Tyr(29) from different protomers are drawn into spatial proximity. Indeed, ring current effects due to the aromatic side chain of Tyr(29) result in the shifting of the Ile(33) methyl protons to high-field positions (0.57 ppm).

(c) H/D Exchange Studies. Hydrogen/deuterium exchange experiments were carried out to determine the amide protons involved in hydrogen-bonding interactions. When solid peptide samples were freshly dissolved in CD₃OD, rapid exchange of most backbone amide protons was observed; an observation previously made in studies on other β-sheet peptides in the literature.[6,19] However, analysis of a TOCSY spectrum recorded approximately 2.5 h after addition of CD₃OD permitted the identification of a number of amide protons that exchange relatively slowly and might hence be involved in hydrogen bond formation. The hydrogen-bonded pairs (N_iH ↔ O_j) thus identified are as follows: R11 ↔ V8, V19 ↔ T16, Q20 ↔ A35, K21 ↔ L14, H23 ↔ T12, Y29 ↔ L26, and I33 ↔ W22. Remarkably, the NH groups of residues Lys(30) and Glu(32) demonstrated maximum resistance to H/D exchange and, indeed, persisted well beyond 24 h. This establishes the strand registry at the dimer interface to be in accordance with design and encourages the belief that the disulfide-bonded antiparallel strands comprising the interface are structurally very well defined.

Conformation of B4dimer in Water. Comparison of the amide/Cᵅ proton correlations, in methanol and water, revealed poorer dispersion in water (especially in the CᵅH region), with much resonance overlap. However, examination of the NOESY spectrum of **B4**dimer in water revealed the encouraging presence of many long-range NOEs connecting residues across designed strands. Cross-strand NH/NH (L26/Y29, F24/C31, W22/I33, L14/K21, T16/V19, Q20/A35), CᵅH/CᵅH (V25/K30, H23/E32 and weaker A13/W22, N15/Q20) (Figure 3b) and $Cᵅ_iH/N_jH$ (V25/C31) NOEs (Figure 4) establish the presence of β-sheet conformation (with the desired registry) in the last hairpin of the **B4** protomer. Strikingly, medium-intensity K30/E32 NH/NH, Y29/I33 CᵅH/CᵅH (Figure 3b), and I33/K30 CᵅH/NH NOEs were observed (Figure 4), confirming the extension of β-sheet conformation across the dimer interface giving rise to a four-stranded β-sheet in water.

A notable feature of the extended eight-stranded β-sheet in methanol was the network of NOEs arising from the interaction of the centrally positioned aromatic residues with other surrounding side chains. Comparison of the appropriate portion of the NOESY spectra of **B4**dimer in methanol and water (Figure 3c,d) revealed that a majority of these interactions were retained in water, specifically between the methyl groups of Ile(33) and the aromatic ring of Tyr(29) across the disulfide-bonded interface.

Structure of B4dimer in Methanol and Water. The NOEs observed in methanol and water are schematically marked on the eight-stranded β-sheet structure shown in Figure 4. A most important point to note is that, for the **B4**dimer peptide in methanol, all the observed NOEs could be accounted for by

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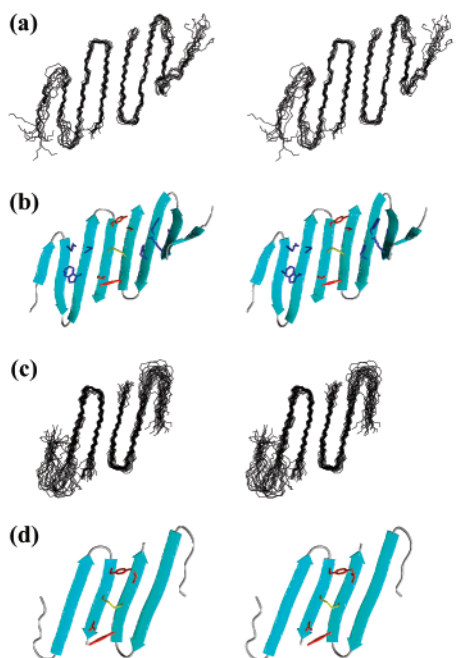
Figure 5. Stereodiagrams illustrating (a) superposition of the 10 best structures calculated for **B4**dimer in methanol, (b) ribbon representation of the mean structure of **B4**dimer, with residues in the hydrophobic cluster (A13, W22, F24) represented as blue sticks. The residues involved in interactions across the dimer interface are in red, and the disulfide bond is in yellow. (c) Superposition of 28 best structures calculated for **B4**dimer in water. (d) Mean structure of **B4**dimer in water with Y29, I33, and the disulfide bond represented as sticks.

considering only one four-stranded β-sheet component together with specific interactions *across the disulfide-bonded dimer interface*. In water, the number of cross-strand interactions are fewer, but clearly the observed NOEs can be rationalized using a two-stranded β-sheet component, together with specific interactions *across the dimer interface*. The choice of sequence permitted unambiguous identification of the NOEs across the dimer interface, confirming the registry of the two antiparallel strands bridged by the disulfide cross-linker.

A total of 446 distance constraints obtained from NMR experiments performed in methanol (398 NOE-based constraints and 48 constraints derived from H/D exchange experiments) and 276 distance constraints obtained from NOESY experiments in water were utilized to perform simulated annealing-based structure calculations using DYANA.[20] It is pertinent to note that despite the presence of C_2 symmetry, **B4**dimer was treated as a 70-residue protein with the appropriate distance constraints, as DYANA does not permit the application of internal symmetry as a restraint in structure calculations.

Figure 5 illustrates the structural assemblies and mean structures obtained for **B4**dimer in methanol [10 superposed structures, rmsd for protomer **1**, 1.24(±0.44) (residues 11−34) and 0.88(±0.33) Å (residues in terminal disulfide-bonded strand); protomer **2**, 1.21(±0.45) (residues 11−34) and 0.84(±0.24) Å (disulfide-bonded strand)] and in water [20 superposed structures, rmsd for residues 20−34, 1.04(±0.32) Å, protomer **1** and 0.91(±0.25) Å, protomer **2**]. Superpositions of the NMR-derived structures were performed using MOLMOL.[21] The calculated structural ensembles indicate that

B4dimer is an extended, eight-stranded, open β-sheet in methanol (Figure 5a). The centrally located aromatic residues (Figure 5b) form local hydrophobic clusters that stabilize the β-sheet. The edge strands of the β-sheet appear frayed toward the termini, consistent with the fact that this region of the β-sheet is most susceptible to solvation invasion. The eight-stranded β-sheet clearly exhibits a right-handed twist as observed in protein β-sheet structures, although the extent of twist is understandably small as compared to those observed in protein β-barrels as exemplified by the porins.[1,16,22]

B4dimer in water (Figure 5c) is a four-stranded β-sheet, flanked by unstructured peptide segments. As in methanol, the β-sheet has an extremely well-defined dimer interface with the requisite β-strand registry and cross-strand side-chain pairing. The hydrophobic cluster comprising residues W22, F24, Y29, and I33 stabilizes the central four β-strands of **B4**dimer and render the β-sheet core resistant to solvent invasion. Figure 5d illustrates the mean orientations of the Y29/I33 pair across the dimer interface.

The deviation of CᵅH chemical shifts from random coil values (called the CᵅH chemical shift index, CSI) is often utilized as an indicator of secondary structure as the direction of the chemical shift change is associated with specific secondary structural elements.[23] Figure 6 illustrates the deviation of the observed CᵅH chemical shifts of **B4**dimer in methanol and in water from their respective random coil values. Clearly, in methanol, all CᵅH chemical shifts (excepting those of residues at designed turns) are significantly shifted to low-field positions, a feature characteristic of β-sheet conformation.[22] The distribution of the CSI in the monomeric unit of the C_2 symmetric **B4**dimer molecule permits demarcation of four strands interspersed by three tight turns. A similar analysis performed for the CᵅH chemical shifts of **B4**dimer in water reveals the presence of positive values of CSI corresponding only to the last hairpin of the **B4** protomer, reflecting the earlier observation that, in water, the β-sheet structure in the **B4**dimer peptide is confined to the central four strands. In addition, the observed deviations are not as large as in methanol, indicating conformational averaging. The low-field positions of the CᵅH resonances of residues flanking designed turns 1 and 2 is noteworthy. Although these turns could not be structurally defined due to the absence of key NOEs, the observed positive CSI for adjacent residues is suggestive of local structure in these regions.

The open sheet structure observed for **B4**dimer in methanol and water could, in principle, aggregate to form larger structures. The formation of large, open aggregates is precluded because of the relatively sharp NMR resonances observed and the fact that gel filtration experiments in water reveal primarily peaks corresponding to monomeric and dimeric structures. The dimerization of **B4**dimer in water could result in the formation of a sandwich structure or a closed barrel, with polar residues on the surface and the nonpolar residues buried in the interior. The

(20) Güntert, P.; Mumenthaler, C.; Wüthrich, K. *J. Mol. Biol.* **1997**, *273*, 283.
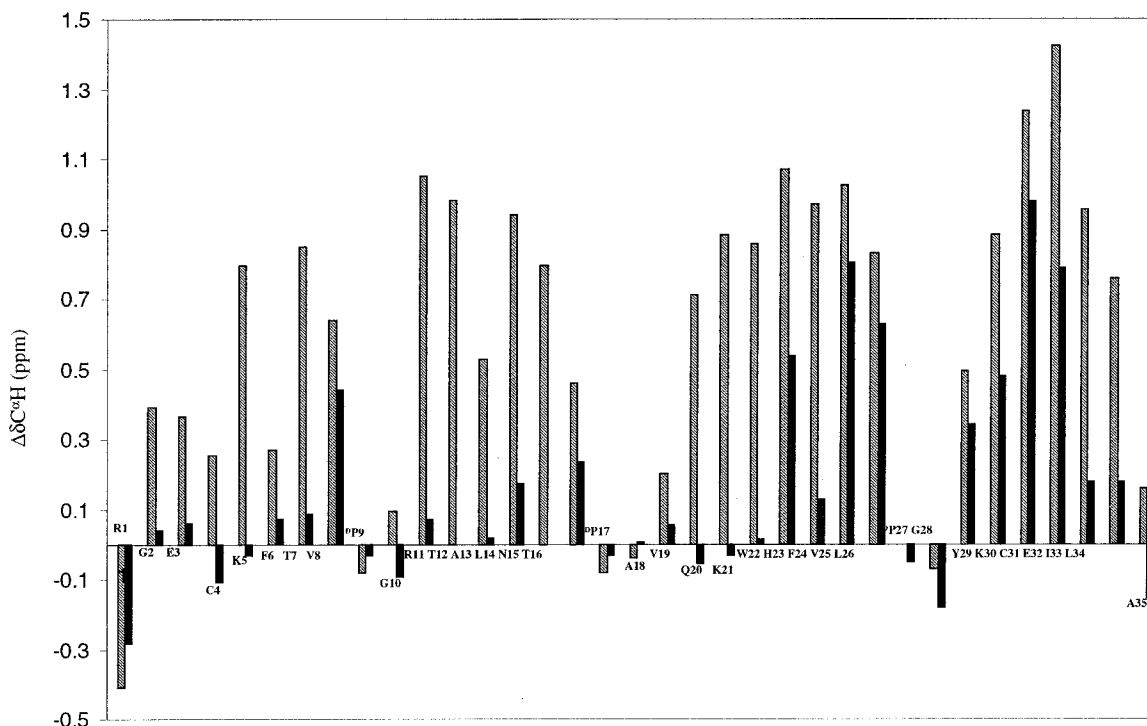(21) Koradi, R.; Billeter, M.; Wuthrich, K. *J. Mol. Graphics* **1996**, *14*, 51.

(22) (a) There are relatively few examples of closed protein β-barrels containing only eight strands. In these cases, the strand lengths are generally larger than that in **B4**cys (and in consequence, **B4**dimer) and the hairpins often contain several loop residues. Indeed, an alternation of tight turns and longer loops is often observed.[16] This permits greater twist of the β-sheet, facilitating closure, with concomitant maintenance of strand registry. (b) For an NMR-derived structure of an eight-stranded β-barrel protein, OmpA, see: Arora, A.; Abildgaard, F.; Bushweller, J. H.; Tamm, L. K. *Nat. Struct. Biol.* **2001**, *8*, 334.
(23) (a) Wishart, D. S.; Sykes, B. D.; Richards, F. M. *Biochemistry* **1992**, *31*, 1647. (b) Wishart, D. S.; Sykes, B. D. *Methods Enzymol.* **1994**, *239*, 363.

Figure 6. Conformational shifts for peptide **B4**dimer in methanol (hatched bars) and water (filled bars). $\Delta\delta C^{\alpha}H$ (ppm) is the deviation of chemical shifts of C^{α} protons from their random coil values (observed $\delta_{C\alpha H}$ − random coil $\delta_{C\alpha H}$). Random coil values are from Wishart et al.[22] The average of the two observed chemical shift values for glycine $C^{\alpha}H$ protons was used.

B4cys sequence was initially designed as an amphipathic sheet. While hydrophobic effects could cause peptide association in water, corresponding solvophobic effects in methanol may be expected to promote association in the case of molecules having large exposed surfaces. Trp(22), which is located near the center of the sheet, could in principle serve as an environmental probe. However, comparison of the intrinsic tryptophan fluorescence of **B4**cys and **B4**dimer revealed identical emission maximums (338 nm, methanol; 348 nm, water), suggesting that the Trp-(22) environment is not perturbed, arguing against the formation of a β-sandwich or barrel. In addition, the absence of defining NOEs relating edge strand residues corroborates the fact that **B4**dimer is an open β-sheet in solution.

We therefore examined the binding of the hydrophobic dye, 8-anilino-1-naphthalenesulfonic acid (ANS), to **B4**dimer in water at pH 3.5. Figure 7 shows a dramatic enhancement and blue shift of ANS fluorescence on addition of **B4**dimer, suggesting appreciable binding of the dye to the open β-sheet structure. Interestingly, dye binding was also observed to the monomer, which is, however, extensively aggregated in aqueous solution. Dye binding in hydrophobic environments created between peptide molecules may account for the enhanced fluorescence. The presence of several positively charged residues in the **B4**cys sequence may also facilitate ANS binding.

Conclusions

An open, eight-stranded β-sheet conformation has been characterized in a designed 70-residue peptide with a C_2 symmetric structure. The successful stabilization of antiparallel β-strands at the interface of two four-stranded β-sheets was achieved by disulfide bridging. In water, the integrity of the four central strands is maintained, undoubtedly, as a result of the presence of the covalent disulfide bridge and local clusters
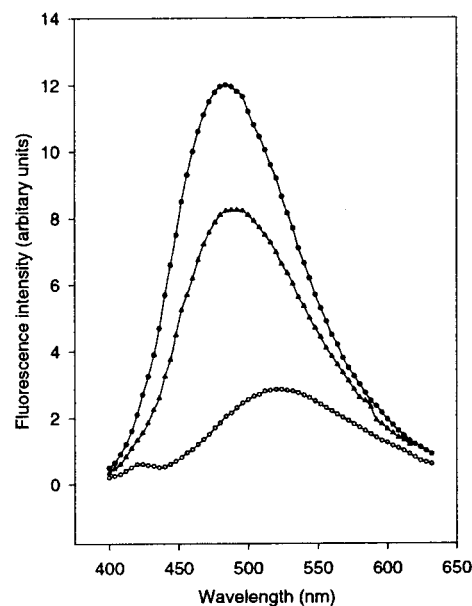

Figure 7. Fluorescence emission spectra of ANS in the absence of peptide (○) and in the presence of 5 μM **B4**dimer (●) and 2 mM **B4**cys (▲). ANS concentration 20 mM; pH 3.5. The excitation wavelength for ANS was 370 nm.

of hydrophobic residues. The fraying of the outer strands of the structure is consistent with the easy access that water molecules have for hydrogen-bonding sites on the backbone of a largely open sheet. The careful choice of sequence has permitted characterization of the interface NOEs in the C_2 symmetric structure. The use of disulfide bonds positioned close to the center of antiparallel strands provides a simple strategy for structure stabilization. Further elaboration of the structure will require removal of the Acm protecting group on Cys(4)

and subsequent oxidation to monomeric and oligomeric products. This task remains to be accomplished.

Experimental Section

Peptide Synthesis and Purification. Peptides **B5**cys and **B4**cys were synthesized on a LKB-Biolynx 4175 semiautomatic peptide synthesizer using standard 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry and activated OPfp esters of protected amino acids. The peptides were assembled on PAC-PEG-PS resin (Perspective Biosystems Inc.) and simultaneously cleaved off the resin and deprotected using a cleavage cocktail of 94% trifluoroacetic acid (TFA), 5% anisole, and 1% ethanedithiol (EDT). After 3–3.5 h, the resin was filtered off, the TFA was removed by evaporation in vacuo, and the peptide was precipitated with ether. The precipitate was repeatedly washed with ether and purified by reversed-phase high performance liquid chromatography (HPLC) on a C_{18} column (5–10 μm, 7.8 mm $\times$ 250 mm) using water/acetonitrile/TFA gradients. The purity of the peptides was analyzed using electrospray ionization mass spectrometry (ESI-MS) on an Hewlett-Packard LCMSD mass spectrometer (M_{calc} of **B5**cys, 4325.0, M_{obs} = 4325.0; M_{calc} of **B4**cys, 4003.6, M_{obs} = 4004.0).

Disulfide Bond Formation. Dimeric forms of peptides **B5**cys and **B4**cys were generated by disulfide bond formation involving the single free cysteine residue [**B5**cys, Cys(34); **B4**cys, Cys(31)]. High-concentration ($\sim$5–10 mM) peptide solutions in methanol were allowed to stand for three weeks to one month to form disulfide cross-links by aerial oxidation. Typically, each oxidation reaction was performed at a time on $\sim$4-mg samples of precursor peptides dissolved in 100–200 μL of methanol. The dimerization reaction was monitored using ESI-MS. Faster reaction rates were achieved by the addition of 20% DMSO to the above solutions, when oxidation was achieved in 5–7 days. The disulfide-bonded dimeric molecules, **B5**dimer and **B4**dimer, were purified using HPLC, and the purity of the peptides estimated using ESI-MS [M_{calc} of **B5**dimer, 8648.0 (2M_{B5cys} − 2[H]), M_{obs} = 8648.0; M_{calc} of **B4**dimer, 8005.2, M_{obs} = 8005.0].

Circular Dichroism. Circular dichroism spectra were recorded on a Jasco J-715 CD spectropolarimeter. Spectra were recorded between 250 and 195 nm (0.1-cm cell) at 0.1-nm intervals with a time constant of 4 s at room temperature, a scan speed of 20 nm/min, and averaged over four separate scans. The spectra obtained were baseline corrected and smoothed. Peptide concentrations were determined using the molar extinction coefficient of Tyr ($\sim$1420 M^{-1} cm^{-1})/Trp($\sim$5600 M^{-1} cm^{-1}).

NMR Experiments and Structure Calculation. NMR experiments were performed at 1 mM peptide concentrations in CD_3OH (300 K) or 90:10 (v/v) H_2O/D_2O (pH 3.5, 323 K). 1D, TOCSY, DQF-COSY, and NOESY (200 ms) experiments were performed on a Bruker DRX500 instrument. Information about amide protons involved in hydrogen bonding was obtained by performing hydrogen exchange experiments in CD_3OD. Solid peptide samples were freshly dissolved in CD_3OD, and quick TOCSY experiments were performed to identify slow-exchanging protons. Additional data sets in CD_3OD and 99.98% D_2O were also collected for the **B4**dimer peptide to obtain distance constraints involving non-amide protons. NOEs were used to derive upper and lower distance constraints by visual segregation into strong (2.0–2.5 Å), medium (2.5–3.5 Å), and weak (3.5–4.5 Å). Structure calculation and simulated annealing was performed using DYANA[19] with 100 random starting structures for 10 000 (structure of **B4**dimer in water) and 30 000 (structure of **B4**dimer in methanol) annealing steps.

Fluorescence. Fluorescence spectra were recorded on a Hitachi 650-60 spectrofluorometer, using excitation and emission band-pass of 10 nm. In ANS-binding studies, the ANS concentration was 20 μM. The excitation wavelength for ANS was 370 nm.

Coordinates. The coordinates have been deposited in the Protein Data Bank (accession codes: **B4**dimer in methanol, 1JY4; **B4**dimer in water, 1JY4).

Supporting Information Available: ^{1}H NMR chemical shift data for **B4**dimer in methanol and water. See any current masthead page for ordering information and Web access instructions.

JA0174276