Interactions Contributing to the Formation of a β-Hairpin-like Structure in a Small Peptide[†]

Volker Sieber and Gregory R. Moe*

Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716

Received March 24, 1995; Revised Manuscript Received August 25, 1995[⊗]

ABSTRACT: A 12 amino acid peptide, model BB, was designed to adopt a β-hairpin tertiary structure in water that might be stabilized by a variety of local, nonlocal, polar, and nonpolar interactions. The conformational properties of model BB with and without an intramolecular disulfide bond (BB-O and BB-R, respectively) were characterized by NMR and CD spectroscopy. The set of observed short- and medium-range nOes were consistent with the formation of stable β-hairpin-like structures by both BB-R and BB-O. BB-O adopts two distinct conformations that differ from each other in the designed reverse turn segment. A reasonably well-defined set of structures was obtained by using restraints from the NMR data in distance geometry calculations. None of the β-hairpin-like structures contain a β-sheet hydrogen bonding network. The distinctive feature of intrastrand and cross-strand pairing of threonine residues observed in all of the calculated structures suggests that hydrophobic interactions between the γ-methyl groups of threonine residues may be the structure-determining interaction in model BB. The implications of these results for the formation of β-sheets during protein folding, the aggregation of peptides as β-sheets, and the *de novo* design of independently folding β-hairpin-like peptides are considered.

β-Sheets are major structural elements of many globular proteins. β-Sheets may also be important early intermediates in protein folding since the extended conformations that are characteristic of β-sheets are near minimum energy conformations of most short segments of polypeptides (Ramachandran & Sasisekharan, 1968) and the formation of β-sheets rapidly restricts the conformational space available to a folding protein (Lifson & Sander, 1980). While much is known about characteristics of β-sheet structures in proteins (Richardson, 1977; Salemme, 1983) and amino acid preferences for β-strand conformations (Fasman, 1967; Chou & Fasman, 1978), there are many questions that remain unanswered about what factors determine the formation and stability of β-sheets. For example, do a particular set of local interactions that favor a reverse turn provide a nucleation site for the extension of a β-sheet hydrogen bonding network (Yao et al., 1994) or are particular properties of residues comprising the β-strands of greater significance in determining the formation of a β-sheet (Minor & Kim, 1994)?

Studies of helical structure have benefited considerably from the availability of monomeric, independently folding peptide models (Kim & Baldwin, 1990). In contrast, peptide models of β-sheets tend to aggregate (Osterman et al., 1984; Osterman & Kaiser, 1985), or do not adopt well-defined conformations (Goodman & Kim, 1990; Blanco et al., 1991, 1993). Recently, Blanco and co-workers have made significant progress in developing several peptide models of two-stranded antiparallel β-sheets (β-hairpin structures). Evidence was obtained from NMR studies for the formation of β-hairpin-like structures by small peptides in water/alcohol mixtures (Blanco et al., 1994a) and in water (Blanco et al., 1993, 1994b). However, it was not possible to determine reasonably defined molecular structures of these peptides or to identify the interactions that were important for stabilizing the β-hairpin-like structures.

We are interested in characterizing the structural and functional properties of small peptides that have the potential for hydrophobic interactions between relatively small surfaces in adjacent segments (Nedved & Moe, 1994; Butcher et al., 1995). These peptides provide experimental model systems for characterizing interactions that may be important in determining the structures of early protein folding intermediates, as well as for developing principles for *de novo* peptide and protein design. For example, we have described a model α-helix−turn−β-strand ($\alpha\beta$) peptide that was designed to be stabilized by intramolecular interactions between hydrophobic residues in adjacent helical and extended segments (Butcher et al., 1995). Although this peptide did not adopt a unique $\alpha\beta$ tertiary structure, the presence of the adjacent extended segment resulted in an increase in helicity, large pK_a shifts of ionizable groups in the helical segment, and slow hydrogen/deuterium exchange rates for nearly all of the backbone amide groups. The structure-promoting effects of intramolecular hydrophobic interactions in this small peptide led us to consider the possibility that similar principles might be employed in the design of a β-sheet-containing model peptide. In particular, we were interested in characterizing the relative contributions of potential polar and nonpolar interactions in stabilizing β-hairpin tertiary structure in a small peptide.

A model peptide, model BB, was designed to adopt a β-hairpin structure having four threonine residues on one face and the Asp, His, and Ser residues of serine protease active sites on the other (Figure 1). Model BB also contains two D-cysteine residues at the amino- and carboxyl-terminal ends to further stabilize β-hairpin structure through formation of a disulfide bond. In the following paragraphs, we describe

[†] Supported by a grant from the Howard Hughes Medical Institute Undergraduate Biological Sciences Education Program (V.S.).

* To whom correspondence should be addressed. Phone: (302)-831-1035. FAX: (302)831-6335. E-mail: moe@brahms.udel.edu.

[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1995.

0006-2960/96/0435-181$12.00/0

the structural properties of model BB with and without an intramolecular disulfide bond (designated as BB-O and BB-R for oxidized and reduced model BB, respectively)[1] as determined by CD and two-dimensional ^{1}H NMR spectroscopy and evaluate the structure-determining interactions suggested by the data.

MATERIALS AND METHODS

Peptide Synthesis. Model BB (Figure 1) was synthesized on a Millipore Excell 9400 peptide synthesizer using Fmoc amino acids (Millipore, Bedford, MA) on a PAL support (Millipore). The peptide was acetylated at the amino terminus using a solution of 1% (w/v) acetic anhydride in DMF before cleaving the peptide from the resin and removal of all side chain protecting groups in a solution of trifluoroacetic acid, anisole, thioanisole, ethanedithiol, and water (85:2.5:5:2.5:5). The peptide was purified by reverse-phase HPLC (Aquapore RP-300, Rainin, Woburn, MA) using a linear gradient of 5−45% acetonitrile in 0.1% trifluoroacetic acid over 30 min. The masses of purified BB-R and BB-O determined by FAB mass spectrometry, 1368.5 and 1366.5 Da, respectively, were identical to the theoretical values.

To obtain the reduced form of the peptide, 10 mg of crude peptide was dissolved in 500 μL of 0.1 M Hepes buffer, pH 8.1, containing a 10-fold molar excess of dithiothreitol and heated to 55 °C for 2 h. It was then purified by reverse-phase HPLC as described above. BB-R remains reduced in solution over a period of weeks as long as the pH is less than 7. The oxidized monomer and dimer peptides were obtained from a dilute solution (1 mg/mL) of purified reduced peptide that was allowed to oxidize at ambient temperature in 0.1 M Hepes buffer, pH 8.1, over a period of 24 h or oxidized directly with $K_3Fe(CN)_6$. After oxidation, the peptide was repurified by reverse-phase HPLC.

Gel Filtration Chromatography. A 0.5 cm × 46 cm column of Sephadex G-25SF was equilibrated with 3 mM sodium acetate buffer, pH 4.0, containing 50 mM NaCl; 50 μL of a 1 or 5 mM solution of BB-R or BB-O was loaded onto the column and eluted with the same acetate buffer. The concentration of peptide in the peak fraction (1 mL) ranged from ∼50 to ∼150 μM depending on the concentration of the solution loaded onto the column. The void volume of the column was determined using Blue Dextran, and a standard curve was constructed from measured elution volumes of peptides having molecular weights of 650, 1520, 2280, and 3500. The peptide standards all exhibit a single CD band at 198 nm that is characteristic of random coil conformations.

CD Spectroscopy. The CD spectra of reduced and oxidized model BB in 3 mM sodium citrate/phosphate/borate buffer, pH 4.0, were recorded using a Jasco J-710 circular dichroism spectrometer that was calibrated with (+)-10-camphorsulfonic acid as described by Johnson (1990). The sensitivity was set at 8 s with a scan rate of 50 nm/min at a step size of 0.1 nm. Each spectrum is the average of three scans which were corrected by subtracting a spectrum of the appropriate solution in the absence of peptide recorded under identical conditions. The spectra shown in Figure 6 are smoothed. Circular quartz cells having a path length of 0.1 cm were used, and the temperature of the samples was maintained at 298 K with a circulating water bath. Peptide concentrations in stock solutions were determined spectrophotometrically using an extinction coefficient of 1490 cm^{-1} M^{-1} at 275 nm for tyrosine in 6 M guanidine hydrochloride.

The effect of peptide concentration on the CD spectrum for BB-R and BB-O was determined by recording spectra from 210 to 250 nm at five peptide concentrations between 60 μM and 4.3 mM. The effect of the salt concentration on the CD spectrum was determined by recording the CD spectrum of each form of model BB from 210 to 250 nm in 3 mM sodium citrate/phosphate/borate buffer, pH 4.0, containing 1 M NaCl. The CD spectra of BB-R and BB-O were also monitored as a function of pH.

NMR Spectroscopy. The 2D NMR experiments were performed on a Bruker AMX-500 spectrometer with a ^{1}H frequency of 500.139 Hz. The concentrations of reduced and oxidized model BB samples used were 8.8 mM in 80% H_2O/20% D_2O and 10 mM in 83% H_2O/17% D_2O, respectively. The pH of both samples was adjusted to 4.0 (uncorrected for isotope effect) with dilute HCl and NaOH in H_2O, using a combination electrode to monitor the pH. Confirmation that BB-R remained reduced during the NMR experiments was obtained by measuring the absorbance at 412 nm after reacting an aliquot of the sample with Ellman's reagent in 50 mM Hepes buffer, pH 8.0, before and after each experiment. DSS was used as an internal reference set at 0.0 ppm. All experiments were acquired with the transmitter set on the H_2O resonance. Solvent suppression was achieved by irradiation of the H_2O resonance during the delay period. Phase-sensitive (States et al., 1982) TOCSY (Bax & Davis, 1985a), ROESY (Bax & Davis, 1985b), and COSY (Derome & Williamson, 1990) experiments were recorded at 293 K. The spectral width was 6024.1 Hz, and 2048 complex data points for each of 512 t_1 values were acquired with 16 scans and 8 dummy scans for each increment. The TOCSY and ROESY experiments used mixing times of 75 and 150 ms, respectively. Data processing was performed on a Silicon Graphics Indigo workstation using FELIX (Biosym, San Diego, CA). Prior to Fourier transformation, the FID was multiplied by an exponential function in both dimensions. The decoupler offset was removed by base line correction. The solvent peak was removed by convolution with a sine bell function. To eliminate t_1 ridges, the spectrum belonging to the first t_1 value was divided by two before Fourier transformation (Otting et al., 1986). Cross-peak intensities were determined by volume integration. $^3J_{NH\alpha}$ coupling constants were determined from 1D spectra with enhanced resolution by zero-filling the FID to 2048 data points.

Temperature dependence, pH dependence, and H/D exchange rates were determined from 1D spectra recorded on a Bruker WM250 spectrometer, by recording 32, 32, and 16 scans, respectively, of 16K data points, acquired over a spectral width of 3.5 kHz. For the pH titration, the pH of the peptide solution was adjusted to 1.7, 2.1, 2.9, 3.6, 3.9, 4.3, 4.60, 5.60, 6.2, 6.90, 7.6, 7.8, 8.2, 8.50, 8.9, 9.4, 10.1, 10.5, 11.1, and 12.0 with dilute NaOH in D_2O and measured using a pH meter. pK_a values of Asp8, Tyr7, and His10 in BB-O were determined from nonlinear least-squares fits to the Henderson−Hasselbalch equation of the change in peak

[1] Abbreviations: BB-R, reduced model BB; BB-O, oxidized model BB containing an intramolecular disulfide bond; Fmoc, 9-fluorenylmethoxycarbonyl; CD, circular dichroism; TFE, trifluoroethanol; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; NMR, nuclear magnetic resonance; nOe, nuclear Overhauser effect; $d_{\alpha N}(i,j)$, $d_{NN}(i,j)$, etc., intramolecular distance between the protons C$^\alpha$H and NH, NH and NH, etc., on residues *i* and *j*; H/D, hydrogen/deuterium; RMSD, root mean square deviation.

height at 2.71 ppm, the change in chemical shift of the phenyl ring protons H2 and H6, or the change of the imidazole C2 proton, respectively, as a function of pH. Amide exchange rates were determined by resuspending the lyophilized peptides in 500 μL of D_2O previously adjusted to pH 4.0 (uncorrected for isotope effect).

Structure Calculation. The structures of BB-R, BB-O1, and BB-O2 were calculated using the distance geometry program DGII running under Insight (Biosym). The starting structures were built in an extended conformation and then minimized with steepest descents and conjugate gradients using the CFF91 force field. Hydrogens were added based on a pH of 4.0. Distance constraints were calculated from the cross-peak volumes of the ROESY spectra using the distance of the β-β′ protons of Asp8 as an internal standard (1.75 Å). The validity of this distance standard was confirmed by comparing the distances calculated from the ROESY data to measured distances between the β−β′ protons of Pro6 and the α and δ protons of Val5 and Pro6, respectively. Dihedral constraints were calculated from the $^3J_{NH\alpha}$ coupling constants. Prochiral peaks were either converted to pseudoatoms in the case of identical chemical shift or else assigned *pro-R* and *pro-S*, according to the most reasonable match of distances. Upper bound correction for pseudoatoms was performed according to Wüthrich et al. (1983). The ROESY cross-peak volumes of protons involved in two conformations were corrected to 100%. *J* coupling constants between Cα and amide protons were converted to φ angles by use of the equation $J = 6.7 \cos^2 \phi - 1.3 \cos \phi + 1.5$ (Ludvigsen, 1991). The smallest contiguous range of φ angles was used (Table 3). The process of distance geometry calculation involved subsequently: (1) triangle inequality bound smoothing; (2) prospective metrization; (3) majorization using inverse square range plus squared average weights; and (4) optimization by simulated annealing over 10 000 steps using an initial energy of 400 kcal/mol, a maximal temperature of 200 K, and a step size of 2.8×10^{-13} for BB-O and 2.6×10^{-13} for BB-R. Twenty structures were calculated for each peptide. Root mean square deviations (RMSD) of the distances of the backbone atoms were determined after superimposition of the backbone of all 20 structures.

RESULTS

Design of Model BB. Model BB was designed as a model experimental system for characterizing the relative significance of various interactions in the formation of a two-stranded antiparallel β-sheet (β-hairpin) in a small peptide. Studies of homo and hetero amino acid polymers (Fasman, 1967) and statistical frequencies of amino acids in β-sheets (Chou & Fasman, 1978) suggest that β-branched and aromatic amino acids have an intrinsic steric preference for extended conformations. Based on these data, one aromatic and five β-branched residues were incorporated into the design of model BB (Figure 1). The aromatic residue, Tyr7, also facilitated the accurate and convenient determination of the peptide concentration (Edelhoch, 1967). Four of the β-branched residues are threonine which, in addition to being β-branched, have the potential for both cross-strand hydrophobic interactions between γ-methyl groups and hydrogen bonding interactions between β-hydroxyl groups in a β-sheet structure. These properties are unique to threonine. The special significance of threonine for stabilizing β-sheet structure was evident in recent host−guest experiments where

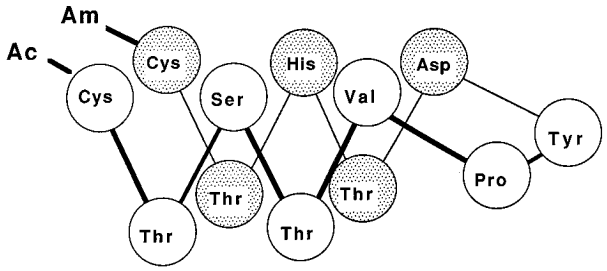

FIGURE 1: Schematic representation of the amino acid sequence of model BB as a β-hairpin tertiary structure. Ac = acetyl; Am = amide.

it was shown that threonine is unique among the naturally occurring amino acids in stabilizing β-sheet structure irrespective of whether it was positioned on the edge or the center of a β-sheet in a small protein (Minor & Kim, 1994a,b). Pairwise interactions between threonine residues were facilitated by positioning two threonine residues separated by one residue in each strand of the β-hairpin. If the peptide forms a β-hairpin, each threonine will be paired with a threonine on the same strand as well as on the adjacent strand, and all four threonine residues will be on the same face of the β-sheet (Figure 1).

Polar interactions between ionized side chains might also be important in β-sheet formation. This possibility was evaluated by incorporating Asp, His, and Ser residues corresponding to the active site residues of serine proteases on the other face of the β-sheet (Figure 1). Trudelle (1982) determined that it would be possible to position these residues on the surface of a β-sheet such that the distances between the side chains would be similar to those observed in the active sites of serine proteases. The Asp and His residues are on the same strand, and Ser is on the adjacent strand. The sequence was designed such that the His^+ imidazolium could form an ion pair with the Asp^- γ-carboxylate and a hydrogen bond with the serine β-hydroxyl group if the peptide adopts a β-hairpin tertiary structure. If polar interactions between these residues contribute to the formation and stability of a β-hairpin structure in the peptide, then the pK_as of the ionizable groups may be shifted from the values observed for the same groups in unstructured peptides [see, for example, Butcher et al. (1995)].

D-Cysteine residues were added to the amino- and carboxyl-terminal ends in order to compare the conformational properties of the peptide with and without an intramolecular disulfide bond constraint. The choice of D-over L-cysteine was based on molecular dynamics studies and experimental results (K. Jacobs, V. Sieber, and G. Moe, unpublished observations) which indicated that a disulfide bond between L-cysteine residues at the terminal ends of the peptide destabilized the β-hairpin structure while a disulfide bond between D-cysteine residues stabilized it. Experimentally we have observed that the L-cysteine analog of model BB does not form an intramolecular disulfide bond under the conditions where the D-cysteine analog is readily oxidized as a monomer. Finally, proline was chosen for its high probability of being found in the second position of a β-turn (Chou & Fasman, 1978). The particular sequence of the turn segment, -V-P-Y-D-, is similar to the sequences of a series of peptides studied by Dyson and co-workers that have been shown to favor reverse turn conformations to varying extents (Dyson et al., 1985, 1988; Yao et al., 1994).

Molecular Mass Determinations. In order to establish whether model BB was monomeric or oligomeric in aqueous

Table 1: Assignments for BB-R at 293 K, pH 4.0

residue	NH	CαH	Cβ H	Cγ H	other
acetyl		2.07			
Cys1	8.41	4.57	2.96/3.06		
Thr2	8.37	4.43	4.34	1.23	
Ser3	8.34	4.55	3.92		
Thr4	8.17	4.36	4.23	1.21	
Val5	8.09	4.42	2.04	0.92	
Pro6		4.40	2.23/1.86	1.99	3.65/3.85
Tyr7	8.04	4.52	3.01		7.12/7.91
Asp8	8.21	4.66	2.66/2.79		
Thr9	8.09	4.37	4.24	1.18	
His10	8.54	4.55	3.24/3.33	7.30	
Thr11	8.18	4.36	4.24	1.21	
Cys12	8.46	4.51	2.93/2.99		
amide	7.16/7.66				

Table 2: Assignments for BB-O1 and BB-O2 at 293 K, pH 4.0

residue	NH	CαH	Cβ H	Cγ H	other
acetyl		2.06			
Cys1	8.48	4.70	3.12/3.24		
Thr2	8.33	4.43	4.35	1.21	
Ser3	8.30	4.54	3.88/3.94		
Thr4	8.13	4.36	4.30	1.19	
Thr4[a]	7.89	4.39	4.17	1.19	
Val5	7.90	4.45	2.05	0.88	
Val5[a]	8.02	4.10	1.96	0.89	
Pro6		4.34	1.81/2.16	1.97	3.62/3.80
Pro6[a]		4.64	2.08/2.30	1.71/1.89	3.50
Tyr7	7.80	4.49	3.05		6.83(H3,5), 7.11(H2,6)
Tyr7[a]	8.43	4.52	3.05		6.79(H3,5), 7.11(H2,6)
Asp8	7.99	4.65	2.73/2.84		
Asp8[a]	8.22	4.67	2.72/2.84		
Thr9	8.06	4.36	4.22	1.17	
Thr9[a]	8.39	4.36	4.33	1.19	
His10	8.49	4.57	3.26/3.36	7.30(H4), 8.58 (H2)	
Thr11	8.08	4.37	4.21	1.20	
Thr11[a]	7.97	4.36	4.26	1.20	
Cys12	8.54	4.68	3.04/3.30		
Amide	7.24/7.69				

[a] Assignments for BB-O2.

solution, its molecular mass was determined by gel filtration chromatography as described under Materials and Methods. The molecular masses calculated for model BB in the reduced (BB-R) and oxidized (BB-O) form were 1370 and 1510 g/mol, respectively, which are in good agreement with the theoretical molecular mass of 1370 g/mol for the monomeric peptides. It was a concern that it might not be possible to distinguish between a monomer and a dimer if the peptide adopts a relatively rigid conformation whose effective volume is determined by the long axis of the β-hairpin. However, the molecular mass determined for a covalently linked dimeric form of BB-O (2540 g/mol) using the same gel filtration column was also in good agreement with the expected molecular mass of the dimer. At concentrations greater than 10 mM, BB-O was observed to aggregate, as indicated by clouding of the solution and changes in the CD and NMR spectra, when the pH was equal to the p*I* of the peptide (∼5.5) but not at pH values ∼1 pH unit above or below the p*I*. There were no indications that BB-O aggregated under the conditions used in either the CD or the NMR experiments.

NMR Studies. The complete assignments for BB-R and BB-O are given in Tables 1 and 2. BB-O exhibits two sets of proton resonances for residues 4−9 and 11 in a ratio of 3:2 (Table 2, Figure 2). This indicates that the disulfide bond-containing peptide adopts two stable conformations or ensembles of closely related conformations (designated as BB-O1 and BB-O2) that interconvert slowly on the NMR time scale. A transient intermediate in the interconversion of the two forms may be indicated by the small peak around the *cis*-Pro CαH region. The integral of this peak is about 1% of the combined integrals of the Pro CαH peaks of BB-O1 and BB-O2. Other than this small peak, there was not a significant population of conformers containing *cis*-proline. With the exception of Thr11 which is next to sterically constrained cystine 1−12, the residues that have different chemical shifts in the two conformations correspond to the designed turn segment of the peptide (residues 5 through 9). Significant chemical shift differences of the phenolic ring proton resonances of Tyr7 between BB-O1 and BB-O2 were also observed (Table 2).

Several NMR parameters of BB-R and BB-O are consistent with extended peptide conformations but not a β-sheet structure. For example, the $^3J_{NH\alpha}$ coupling constants for many of the residues in the β-strand segments of BB-R and BB-O1/O2 have values that are nearer the value of 9 expected for extended conformations than the value of 4 expected for helical conformations (Table 3). However, the CαH chemical shifts for BB-R and BB-O1/O2 were not significantly different from random coil values (Table 3). The Cα protons of residues in β-sheets are generally observed to be shifted downfield by more than 0.1 ppm relative to random coil values (Wishart et al., 1993). Also, several of the amide proton temperature coefficients for BB-O1 and BB-O2 (Table 3) were relatively small (<8 ppb/K) and exhibit a magnitude variation with a periodicity of two residues in each of the designed β-strand segments. Although relatively small values for the temperature coefficients (<6 ppb/K) are regarded as indicative of strong hydrogen bonding interac-

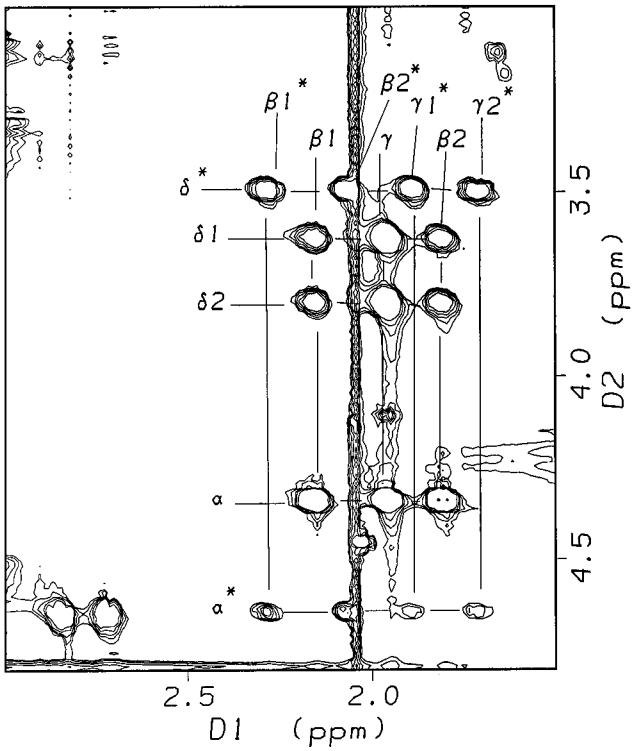

FIGURE 2: Portion of a 500-MHz TOCSY spectrum of 10 mM oxidized model BB (BB-O) at 293 K, pH 4.0, showing the distinct set of resonances for the two conformations of Pro6.

Table 3: Comparison of Δδ CαH Values[a] and $^3J_{NH\alpha}$ Coupling Constants for BB-R, BB-O1, and BB-O2 and Amide Proton Temperature Coefficients for BB-O1 and BB-O2

	Δδ CαH (ppm)			$^3J_{N\alpha}$ (Hz)			−Δδ/Δ*T* (ppb/K)	
residue	BB-R	BB-O1	BB-O2	BB-R	BB-O1	BB-O2	BB-O1	BB-O2
Cys1				6.0	9.0	9.0	5.2 ± 0.4	5.2 ± 0.4
Thr2	0.08	0.08	0.08	9.0	6.2	6.2	4.1 ± 0.2	4.1 ± 0.2
Ser3	0.5	0.04	0.04	7.5	7.4	7.4	ND[b]	ND
Thr4	0.01	0.01	0.04	9.0	8.5	8.5	3.7 ± 0.4	3.7 ± 0.4
Val5	0.24	0.26	−0.08	7.0	8.5	8.0	8.0 ± 0.4	8.0 ± 0.4
Pro6	−0.07	−0.13	0.17					
Tyr7	−0.08	−0.11	−0.08	7.0	6.0	9.0	8.2 ± 0.4	5.3 ± 0.3
Asp8	−0.11	−0.12	−0.10	6.0	13.5	5.8	ND	ND
Thr9	0.02	0.01	0.01	7.0	6.4	6.0	7.6 ± 0.7	7.6 ± 0.7
His10	−0.08	−0.06	−0.06	9.0	7.2	7.2	4.6 ± 0.4	4.6 ± 0.4
Thr11	0.01	0.02	0.01	6.5	8.8	7.0	6.0 ± 0.4	6.0 ± 0.4
Cys12				6.0	7.2	7.2	4.5 ± 0.1	4.5 ± 0.1

[a] Random coil chemical shifts used were from Bundi and Wüthrich (1980). [b] ND, not determined.

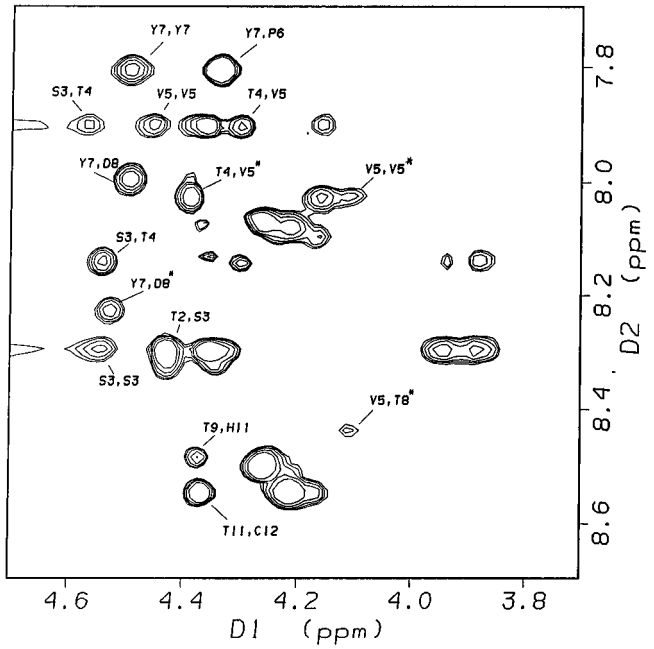

FIGURE 3: Portion of a 500-MHz ROESY spectrum of 10 mM oxidized model BB (BB-O) at 293 K, pH 4.0, showing the sequential compared to intraresidue $d_{\alpha N}$ nOes.

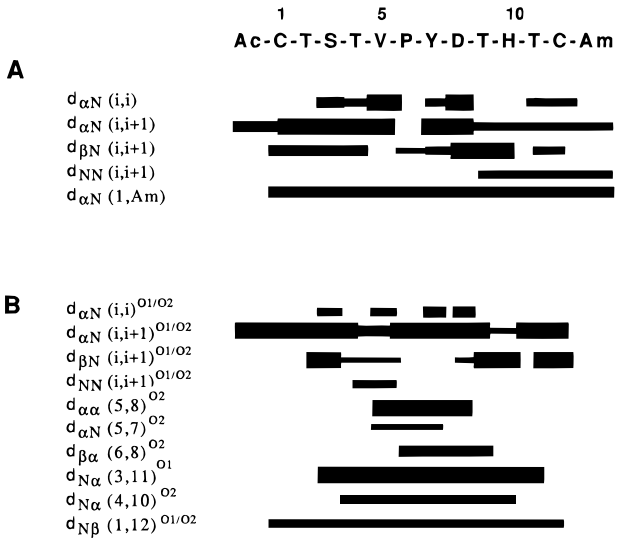

FIGURE 4: Summary of the nOes observed for reduced (A) and oxidized (B) model BB (BB-R and BB-O, respectively) at 293 K that were used in the distance geometry calculations. The superscripts O1 and O2 in (B) indicate that the connectivities were observed for conformations O1 and O2, respectively, of oxidized model BB. The thickness of the lines is proportional to the integrated volume of the ROESY cross-peak.

tions with the amide proton (Rose et al., 1985), the periodicity observed is inconsistent with the periodicity expected for any of the possible regular β-sheet hydrogen bonding networks involving these residues. Alternatively, the observed pattern variation of the coefficients might be explained by an irregular main chain−side chain hydrogen bonding network or, more likely, strong main chain−side chain hydrogen bonds.

Unequivocal evidence that both BB-R and BB-O1/O2 adopt stable β-hairpin-like conformations in water was obtained from the ROESY experiments. Both reduced model BB and oxidized model BB exhibit intense sequential $d_{\alpha N}$-(i,i+1) and $d_{\beta N}$(i,i+1) nOes compared with intraresidue $d_{\alpha N}$-(i,i) nOes at short mixing times (Figures 3 and 4). Formation of an intramolecular disulfide bond in BB-O1/O2 results in a general increase in the intensity of sequential nOes and a decrease in the intensity of d_{NN} nOes (Figure 4) which is consistent with the expected increased stability of a β-hairpin tertiary structure when the ends of the peptide are linked by a covalent bond. Finally, unambiguous cross-strand nOes were observed for both BB-R and BB-O1/O2, clearly showing that the amino- and carboxyl-terminal segments of both the reduced and oxidized forms of the peptide fold back on each other (Figure 4).

Using the covalent structure of the peptide, $^3J_{NH\alpha}$ coupling constants, and distance restraints calculated from nOe cross-peak volumes in a distance geometry calculation, it was possible to define the solution conformations of BB-R, BB-O1, and BB-O2 more precisely. Most of the medium-range nOes were between backbone protons, and as a result, many of the side chain conformations are poorly defined. However, the restraints were sufficient to define the amide backbone of both peptides as β-hairpin-like structures comprised of two twisted β-strands connected by a reverse turn of variable conformation (Figure 5). Similar structures were obtained when the restraints were relaxed by grouping the nOes into strong (<2.5 Å), medium (2.5−3.5 Å), and weak (>3.5 Å) categories.

The RMSD of the backbone heavy atoms of 20 structures calculated for each data set are 1.98 Å (BB-R), 1.20 Å (BB-O1), and 1.72 Å (BB-O2). These values are relatively small considering the potential for conformational averaging in a small peptide and the limited possibilities for obtaining nOe restraints in model BB. Most of the variation in the

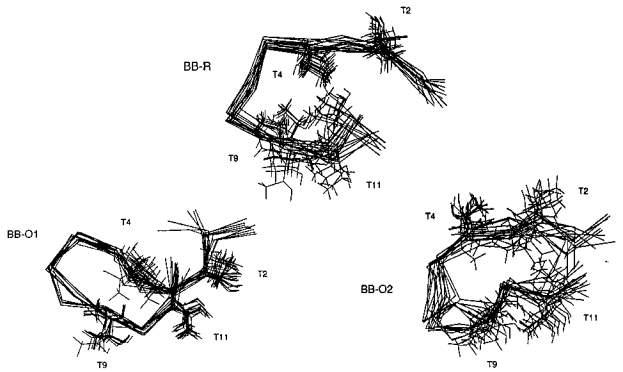

FIGURE 5: Comparison of the structures obtained for reduced model BB (BB-R) and the two conformations of oxidized model BB (BB-O1 and BB-O2) using the NMR restraints in distance geometry calculations. Only the Cα trace and side chains of Thr2, Thr4, Thr9, and Thr11 are shown. For purposes of clarity, only 18 of 20, and 16 of 20 structures of BB-O1 and BB-O2, respectively, are shown.

calculated structures of BB-R and BB-O1 occurs in particular segments. For example, the region around His10 in BB-R is poorly defined as is the N-terminal acetyl group of BB-O1. Despite the fact that the largest number of medium-range nOes was observed for BB-O2 (Figure 4), the structures calculated exhibit the greatest overall variation.

The ϕ,ψ angles for individual structures as well as the average values for all structures correspond to sterically low-energy regions of ϕ,ψ space for L-amino acid residues (Ramachandran & Sasisekharan, 1968) although several, ϕ,ψ values for BB-R and BB-O2 structures are near usual left-handed helical and twisted β-sheet regions. The orientations of the β-strands in calculated BB-R and BB-O1 structures are similar to each other and are approximately related to those in BB-O2 by a 180° rotation of each β-strand—essentially, it is as if BB-O1 were twisted inside-out. The β-strands of BB-O1 are twisted to a much greater extent than they are in BB-R or BB-O2. The larger twist results in BB-O1 having a boat shape configuration with cross-strand pairs of Thr2 with Thr11 and Thr4 with Thr9 on one side (Figure 5). The turn segments of BB-R and BB-O2 are much wider than the turn segment of BB-O1.

None of the calculated structures was observed to have a β-sheet hydrogen bonding network nor did they exhibit a hydrogen bonding pattern that is characteristic of any particular type of β-turn. Only a small subset of the $d_{\alpha\alpha}$, d_{NN}, and $d_{N\alpha}$ nOes expected for a β-sheet structure was observed for BB-R and BB-O1/O2, and the set of cross-strand nOes was different for each form. As a further test of possible cross-strand hydrogen bonding between main chain amide groups, the amide proton H/D exchange rates were determined for BB-R and BB-O1/O2 at 4 °C (BB-O1/O2 only) and 25 °C, pH 4.0. The observed rates were not significantly different from calculated rates for unstructured peptides under the same conditions (Bai et al., 1993). These observations are consistent with the conclusion based on chemical shifts and amide proton temperature coefficients that the β-hairpin-like conformations of BB-R and BB-O1/O2 are not stabilized by a β-sheet hydrogen bonding network.

Although the peptides adopt β-hairpin-like conformations, there does not appear to be any structure-stabilizing interactions between the side chains of Ser3, Asp8, and His10. There were no nOes observed that might indicate that the residues are in close proximity to each other. Also, there were no shifts in the pK_a values of the ionizable groups that might indicate strong interactions with other polar groups. The pK_a values for Asp8, His10, and Tyr7 in BB-O1/O2 of 4.1 ± 0.0, 6.8 ± 0.3, and 10.2 ± 0.1 determined by NMR at either 277 K or 298 K are essentially the same as those observed in random coil peptides under similar conditions (Bundi & Wüthrich, 1979). The pK_a values of the ionizable groups in BB-R were not determined due to complications of cysteine oxidation during the titration.

Although it was not possible to unequivocally detect interactions between threonine residues in the ROESY experiments due to the lack of chemical shift dispersion, intrasegment and/or cross-strand pairing of threonine residues was evident in all of the calculated structures (Figure 5). In fact, the feature that distinguishes the structures of BB-R and BB-O1 from BB-O2 is that the BB-R and BB-O1 structures have cross-strand threonine pairs while those of BB-O2 have only intrastrand threonine pairs. While both hydrophobic interactions between γ-methyl groups and hydrogen bonding of β-hydroxyl groups are potential contributors to threonine pairing, no consistent pattern of side chain−side chain or side chain−main chain hydrogen bonds was observed in any of the calculated structures. Hence, the observed pairing of threonine residues in the calculated structures was due entirely to the backbone conformations defined by the NMR restraints.

CD Studies. The CD spectrum of BB-R is characterized by a broad, weak minimum centered at 199 nm. Except for the fact that the magnitude of the band is relatively small, this type of spectrum is usually attributed to random coil conformations (Woody, 1985). Although the peptide is not a random coil, this interpretation is consistent with the calculated structures in which there was no hydrogen bonding network, and, therefore, it would not be expected to exhibit a β-sheet spectrum. The relatively small magnitude of the minimum at 199 nm may be associated with the observed extended amide backbone conformations since peptides that are forced, by the presence of a proline repeat in the sequence, to have random coil conformations that are dominated by turns exhibit a CD spectrum having a minimum at the same wavelength but of considerably greater magnitude (Nedved et al., 1994). A CD spectrum similar to that of BB-R has been observed for a 16 residue peptide fragment of protein G that was shown by Blanco et al. (1994b) to exhibit similar conformational dynamics by NMR.

The CD spectrum of BB-O is quite different than that of BB-R (Figure 6). It is characterized by very weak bands at 220 and 200 nm. Antiparallel β-sheets typically exhibit a minimum at 216 nm and a maximum near 200 nm (Woody, 1985). Since neither reduced nor oxidized model BB appears to form a β-sheet hydrogen bonding network, the differences between the reduced and oxidized forms must be due to other contributions to the CD spectrum. For example, it is likely that the contributions from the reverse turns, Tyr7, and the disulfide bond are quite different in the two forms of the peptide.

The CD spectra of BB-R and BB-O are independent of peptide concentration from 60 μM to 4.3 mM ($[\theta]_{210} = -6600 \pm 200$ deg·cm^2/dmol for BB-R and $[\theta]_{220} = -4700 \pm 300$ deg·cm^2/dmol for BB-O) and independent of salt concentration up to 1 M NaCl. The former observation provides additional evidence that the peptides are monomeric. The CD spectra of BB-R and BB-O1/O2 were dependent on pH, and it was possible to follow the ionization of Tyr7, Asp8, and His10 through changes in the CD spectrum.

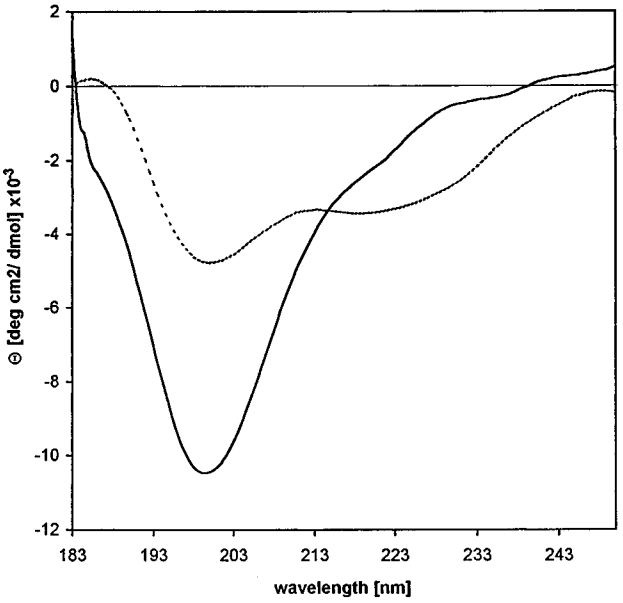

FIGURE 6: Comparison of the CD spectra of reduced (solid line) and oxidized model BB (dashed line) in 3 mM sodium citrate/phosphate/borate buffer, pH 4.0 at 293 K. The spectra are smoothed.

However, the pH-dependent changes in the CD spectrum were too small ($\Delta[\theta] < \sim 1000$ deg·cm^2/dmol) to be used in accurately estimating the pK_a values.

DISCUSSION

A 12 amino acid peptide, model BB, was designed to adopt a 2-stranded antiparallel β-sheet (β-hairpin) tertiary structure in water. The observation of large sequential nOes compared to small or absent intraresidue $d_{\alpha N}$ and $d_{\beta N}$ nOes, relatively large CαH−NH coupling constants, and several cross-strand nOes for both reduced and oxidized forms of model BB (BB-R and BB-O, respectively) was consistent with the formation of a β-hairpin-like conformation. Two sets of NMR resonance lines for residues in the turn segment were observed for BB-O, showing that the oxidized peptide adopted two distinct conformations or conformational ensembles (BB-O1 and BB-O2) that differed primarily in the conformation of the turn segment and the resulting orientation of the β-strands.

Although conformational averaging and limited chemical shift dispersion precluded the possibility of determining unique structures, it was possible to obtain more precise structural information for both the reduced and oxidized forms of the peptide by using the restraints derived from NMR data in distance geometry calculations. All of the calculated structures were β-hairpin-like (Figure 5), confirming the qualitative interpretation of the NMR data. However, none of the calculated structures contains a β-hairpin-defining hydrogen bonding network. The Cα proton chemical shifts, amide proton temperature coefficients, amide proton H/D exchange rates, and CD spectra confirmed that the absence of a β-sheet hydrogen bonding network in the calculated structures was not an artifact of limited cross-strand nOe restraints.

Why does model BB adopt stable β-hairpin-like structures in water irrespective of whether there is a covalent disulfide bond constraint at the terminal ends of the peptide if there is no main chain hydrogen bonding network? One possibility is that the β-hairpin-like conformations are essentially determined by the local steric and hydrogen bonding interactions within the turn segment. The propensity for a reverse turn might bring the ends of the peptide in close proximity where a variety of transient and weak polar and nonpolar interactions might then be able to stabilize a β-hairpin-like structure. The extensive studies of Dyson and co-workers (Dyson et al., 1988; Yao et al., 1994) have shown that a number of small peptides having sequences similar to that of the model BB turn segment favor, to varying extents, reverse turn conformations. However, the CαH chemical shifts, nOes, and calculated reverse turn conformational ensembles of BB-R, BB-O1, and BB-O2 were different from each other and from those of the most closely related peptides studied by Dyson et al. Furthermore, structure calculations that excluded restraints from cross-strand nOes showed no intrinsic propensity for a β-hairpin-like structure (data not shown). In fact, it appears that Val5, which is not in the peptides studied by Dyson et al., sterically interferes with formation of a tight β-turn that would, presumably, be required to facilitate interactions between the β-strand segments. Clearly, local interactions in the turn segment are an important constraint on the possible conformations adopted by model BB but are not a determining factor for the formation of the β-hairpin-like structures observed.

Ionic interactions between side chains have been shown to stabilize helical structure in peptides (Marqusee & Baldwin, 1987). The possibility that similar effects might be achieved in a β-hairpin peptide was considered by incorporating potential ionic interactions in the design of model BB. Although BB-R and BB-O adopted β-hairpin-like structures, the CD spectra were independent of salt concentration to 1 M NaCl, indicating that there was no significant structure-stabilizing effect of interactions between Asp^-8 and His^+10. The CD spectra were dependent on pH; however, the changes were small and could not be correlated with basis spectra characteristic of regular secondary structure. The pH dependence is probably due to local side chain−main chain hydrogen bonding interactions and changes in the aromatic residue contributions to the CD spectrum.

Another possible explanation for the formation of β-hairpin-like structures by model BB is that they are stabilized by hydrophobic interactions between threonine residues. Intrastrand and/or cross-strand pairing of threonine residues was the only characteristic of the calculated β-hairpin-like structures that was common to BB-R, BB-O1, and BB-O2. The result is striking since there were no restraints in the distance geometry calculation that directly define threonine pairing; instead, it was due entirely to the backbone configurations defined by the restraints. While it is possible that hydrogen bonding between threonine β-hydroxyl groups contributes to threonine pairing, this seems quite unlikely given the severe conformational constraints required for such hydrogen bonds and the failure to observe hydrogen bonding interactions between several more polar side chains.

Interactions between hydrophobic residues have been identified as possible structure-determining factors in other β-hairpin (Blanco et al., 1994b) and β-turn (Yao et al., 1994) model systems. In addition, it has been suggested that hydrophobic interactions between relatively small surfaces are important in capping the ends of helices (Seale et al., 1994), in stabilizing helical structure (Padmanabhan & Baldwin, 1994), and in stabilizing an αβ-tertiary structure in a small peptide (Butcher et al., 1995). As noted above, it has been shown that threonine is somewhat unique in

stabilizing the β-sheet structure at both hydrophobic and solvent-exposed sites (Minor & Kim, 1994b). This suggests that significant hydrophobic interactions involving the threonine side chain are possible. Nevertheless, pairwise hydrophobic interactions between threonine residues, which are generally regarded as hydrophilic residues, in a small peptide are unprecedented, and this explanation, though it is plausible, must be regarded as speculative until the existence of such interactions in model BB can be shown directly.

The observation in this study that the relatively stable β-hairpin-like conformations adopted by BB-R and BB-O1/O2 in water do not appear to be stabilized by main chain hydrogen bonding networks has important implications for protein folding and the aggregation of peptides as β-sheets. Close proximity of the appropriate main chain hydrogen bond donors and acceptors, a condition that is fulfilled by the structures of model BB in water, is apparently insufficient for the formation of a stable β-sheet hydrogen bonding network. Evidently, intramolecular main chain−main chain hydrogen bonds of β-sheets are unstable unless solvent water is excluded. Typically, this appears to be accomplished through the association of hydrophobic amino acid side chains since hydrophobic amino acids are observed to have a statistical preference for β-sheet structure (Chou & Fasman, 1978; Lifson & Sander, 1979) and, conversely, when β-sheets occur in proteins they are often observed to be part of the hydrophobic core.

If hydrophobic interactions between γ-methyl groups of threonine residues are in fact the structure-determining interactions for model BB, the surfaces involved are apparently too small to have a significant effect on desolvating the amide backbone. The balance of hydrophobicity and hydrophilicity in model BB is probably critical in allowing the peptide to fold into β-hairpin-like conformations yet avoid the oligomerization and aggregation that are often observed with β-sheet-forming peptides (Osterman et al., 1984, 1985). Further studies of model BB and related analogs should provide greater insight into the formation of β-sheets during protein folding, the aggregation of peptides as β-sheets, and the *de novo* design of other β-hairpin-like peptides.

ACKNOWLEDGMENT

We thank David G. Osterman and Gary F. Musso for their comments on the manuscript. The design of model BB was greatly influenced by earlier unpublished β-hairpin peptide models proposed by David G. Osterman.

REFERENCES

Bai, Y., Milne, J. S., Mayne, L., & Englander, S. W. (1993) *Proteins: Struct., Funct., Genet. 17*, 75−86.
Bax, A., & Davis, D. G. (1985a) *J. Magn. Reson. 63*, 207−213.
Bax, A., & Davis, D. G. (1985b) *J. Magn. Reson. 65*, 355−360.
Blanco, F. J., Jimenez, M. A., Rico, M., Santoro, J., Herranz, J., & Nieto, J. L. (1991) *Eur. J. Biochem. 200*, 345−351.
Blanco, F. J., Jimenez, M. A., Rico, M., Santoro, J., Herranz, J., & Nieto, J. L. (1993) *J. Am. Chem. Soc. 115*, 5887−5888.
Blanco, F. J., Jimenez, M. A., Rico, M., Santoro, J., Pineda, A., & Nieto, J. L. (1994a) *Biochemistry 33*, 6004−6014.
Blanco, J., Rivas, G., & Serrano, L. (1994b) *Struct. Biol. 1*, 584−590.
Bundi, A., & Wüthrich, K. (1979) *Biopolymers 18*, 285−297.
Butcher, D., Bruch, M. D., & Moe, G. R. (1995) *Biopolymers 36*, 109−120.
Chou, P. J., & Fasman, G. D. (1978) *Adv. Enzymol. Relat. Areas Mol. Biol. 47*, 45−147.
Derome, A., & Williamson, M. (1990) *J. Magn. Reson. 88*, 177−185.
Dyson, H. J., Cross, K. J., Houghten, R. A., Wilson, I. A., Wright, P. E., & Lerner, R. A. (1985) *Nature 318*, 480−483.
Dyson, H. J., Rance, M., Houghten, R. A., Lerner, R. A., & Wright, P. E. (1988) *J. Mol. Biol. 201*, 161−200.
Edelhoch, H. (1967) *Biochemistry 6*, 1948−1954.
Fasman, G. D. (1967) *Biological Macromolecules Series 1* (Fasman, G. D., Ed.) pp 499−604, Marcel Dekker, Inc., New York.
Goodman, E. M., & Kim, P. S. (1990) in *Current Research in Protein Chemistry* (Villafranca, J. J., Ed.) pp 301−308, Academic Press, San Diego.
Johnson, W. C. (1990) *Proteins: Struct., Funct., Genet. 7*, 205−214.
Kim, P. S., & Baldwin, R. C. (1990) *Annu. Rev. Biochem. 59*, 631−660.
Lifson, S., & Sander, C. (1979) *Nature 282*, 109−111.
Lifson, S., & Sander, C. (1980) *J. Mol. Biol. 139*, 627−639.
Ludvigsen, S., Andersen, K. V., & Poulsen, F. M. (1991) *J. Mol. Biol. 217*, 731−736.
Marqusee, S., & Baldwin, R. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8898−8902.
Minor, D. L., Jr., & Kim, P. S. (1994a) *Nature 367*, 660−663.
Minor, D. L., Jr., & Kim, P. S. (1994b) *Nature 371*, 264−267.
Nedved, M. L., & Moe, G. R. (1994) *Nucleic Acids Res. 22*, 4705−4711.
Nedved, M. L., Gottlieb, P. A., & Moe, G. R. (1994) *Nucleic Acids Res. 22*, 5024−5030.
Osterman, D. G., & Kaiser, E. T. (1985) *J. Cell. Biochem. 29*, 57−82.
Osterman, D. G., Mora, R., Kezdy, F. J., Kaiser, E. T., & Meridith, S. C. (1984) *J. Am. Chem. Soc. 106*, 6845−6847.
Otting, G., Widmer, H., Wagner, G., & Wüthrich, K. (1986) *J. Magn. Reson. 66*, 187−193.
Padmanabhan, S., & Baldwin, R. L. (1994) *Protein Sci. 3*, 1992−1997.
Ramachandran, G. N., & Sasisekhran, V. (1968) *Adv. Protein Chem. 23*, 284−438.
Richardson, J. S. (1981) *Adv. Protein Chem. 34*, 167−339.
Rose, G. D., Gierasch, L. M., & Smith, J. A. (1985) *Adv. Protein Chem. 37*, 1−109.
Salemme, F. R. (1983) *Prog. Biophys. Mol. Biol. 42*, 95−133.
Seale, J. W., Srinivasan, R., & Rose, G. D. (1994) *Protein Sci. 3*, 1741−1745.
States, D. J., Haberkorn, R. A., & Ruben, D. J. (1982) *J. Magn. Reson. 48*, 286−292.
Trudelle, Y. (1982) *Int. J. Pept. Protein Res. 19*, 528−535.
Wishart, D. S., Sykes, B. D., & Richards, F. M. (1991) *J. Mol. Biol. 222*, 311−333.
Woody, R. (1985) in *The Peptides* (Udenfried, S., Meienhofer, J., & Hruby, V. J., Eds.) Vol. 7, pp 15−114, Academic Press, New York.
Wüthrich, K., Billeter, M., & Braun, W. (1983) *J. Mol. Biol. 169*, 949−961.
Yao, J., Dyson, H. J., & Wright, P. E. (1994) *J. Mol. Biol. 243*, 754−766.

BI950681O