Structure, Folding, and Energetics of Cooperative Interactions between the β-Strands of a *de Novo* Designed Three-Stranded Antiparallel β-Sheet Peptide

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Abstract: The effect of cooperative interactions between β-strands in enhancing β-sheet stability has been examined quantitatively by NMR using rationally designed synthetic peptides [β-hairpin (**2**β) and related 24-residue three-stranded antiparallel β-sheet (**3**β)] which are significantly folded in aqueous solution. The two hairpin components of **3**β show quite different temperature-dependent stability profiles showing that a two-state model for folding (random coil ↔ three-stranded antiparallel β-sheet) is inappropriate. A four-state model for folding, involving intermediate C- and N-terminal β-hairpin conformations, is more consistent with the data. Thermodynamic analysis shows that folding of the C-terminal hairpin of **3**β is entropy-driven, as previously described for the isolated hairpin **2**β, but that the N-terminal hairpin, which is stabilized by a motif of aromatic residues (W4, F6, and Y11), is enthalpy-driven, consistent with stabilization through $\pi-\pi$ interactions that are electrostatic in origin. NOE data, as well as structure calculations, support the formation of this stabilizing motif on one face of the β-sheet. Both hairpins are associated with a significant ΔC_p° for folding, suggesting the burial of hydrophobic surface area as an important contributor to stability. We demonstrate quantitatively, by comparison of data for **2**β versus **3**β, that the folded population of the C-terminal β-hairpin is cooperatively enhanced by the interaction of the third strand.

Introduction

The design of peptides that fold into a predetermined target structure provides a measure of our progress toward elucidating the stereochemical principles that define a unique molecular conformation, and our understanding of the nature of the interactions that stabilize it.[1] The major determinants of conformational stability and specificity in driving a polypeptide chain toward a particular compact folded state are well recognized; however, the relative importance of these different determinants (hydrogen bonding interactions, hydrophobic contacts, and mainchain torsion angle preferences) are still a matter of conjecture.[2] Commitment of a folding polypeptide chain to a particular conformational state or folded topology must depend not just on a network of stabilizing interactions but also on the weakly cooperative interplay between them that favors one conformational state over another. Cooperativity is a ubiquitous phenomenon in biological molecular recognition; however, a quantitative description at the molecular level remains a significant challenge.[3] The cooperative nature of the protein folding transition and the ability of the polypeptide chain to fold to a unique three-dimensional structure are defining characteristics of globular proteins and are usually associated with protein tertiary interactions.[4,5] Cooperativity within isolated elements of secondary structure has already been demonstrated quantitatively for α-helical peptides,[6−8] but model β-sheets have proved less amenable to quantitative analysis. A number of model peptide systems are now beginning to emerge,[9−12] some of which have demonstrated some degree of cooperativity in their ability to fold. The interest in β-sheet structure, folding, and stability has come to the fore in the context of protein folding-related disease states. The role of β-strands in the formation and propagation of amyloid, and the progression of

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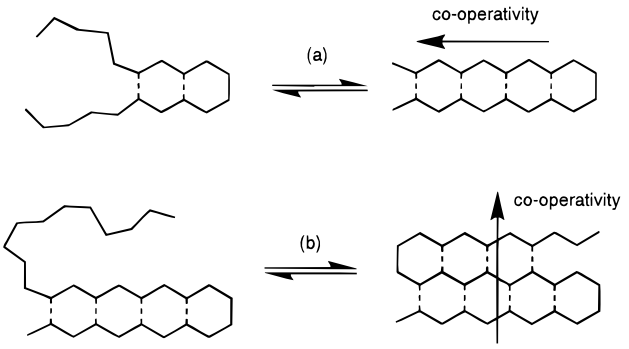

Figure 1. Cooperative interactions in β-sheets parallel (a) and perpendicular (b) to the strand direction.

a variety of pathological disorders,[13−15] suggests that quantitative studies of cooperativity in model systems could provide valuable insight into these processes.

The folding of a multistranded β-sheet structure requires sheet propagation in orthogonal directions both parallel and perpendicular to the strand direction (Figure 1). Here, the extent to which cooperative interactions between β-strands enhances sheet stability has been examined quantitatively by NMR in a model β-hairpin (**2**β) and related three-stranded antiparallel β-sheet (**3**β) using rationally designed synthetic peptides that are significantly folded in aqueous solution. By monitoring the temperature-dependence of the folded population of the two NG type I′ turns we show that the thermodynamic properties of the two constituent β-hairpins that make up **3**β are quite different, in one case entropy-driven and in the other case enthalpy-driven, the latter reflecting edge−face π−π interactions involving a stabilizing motif of aromatic residues introduced in the process of rational design. These data rule out a simple two-state model for folding from random coil to fully folded three-stranded antiparallel β-sheet, but are consistent with a more complex four-state equilibrium that also involves the intermediate C- and N-terminal β-hairpins. On the basis of this model, we demonstrate that hairpin stability is enhanced by the interaction of the third strand by a small but significant incremental cooperative contribution.

Methods

Materials and NMR Methodology. The preparation and purification of peptides has been described in detail previously together with the NMR methodology used.[16,17]

Analysis of Peptide Aggregation. Dilution experiments were carried out to examine the concentration dependence of NMR parameters in the range 30 μM to 2 mM, and over the temperature range 278 to 333 K; at a given temperature, no significant concentration-dependent differences in $\delta^{H\alpha}$ values or line widths were detected for either peptide at pH 3. In the case of **3**β, we observed pH-dependent changes in the NMR spectrum such that at pH 5 line widths were somewhat broader suggesting that two or more conformations may be present in intermediate exchange. The spectrum sharpened considerably at lower pH suggesting that the origin of this effect may arise from titration of the side chain carboxylate group of Glu3. All data were subsequently collected at pH 3 for both peptides. The concentration range was extended by examining CD spectra down as low as 7.5 μM, with no evidence for concentration-dependent folding. Estimates of the folded population of the peptide by CD and NMR at very different concentrations are in reasonable agreement within the limits of experimental errors, indicating that the monomeric state persists over the concentration range studied.

Circular Dichroism. Far-UV CD spectra were recorded on an AVIV model 62DS spectrometer (Aviv associates), using a 0.2 cm path length cell. Stock peptide solutions of 1 mL of 1 mM concentration were subsequently diluted with water or aqueous methanol to give solutions in the concentration range 7.5 to 50 μM. Methanol titration studies by CD used 50 μM solutions of peptide. Typically 10 scans were acquired over the wavelength range 190−250 nm in 1.0 nm steps using a bandwidth of 4 nm at 293 K. The resulting data were smoothed, and baseline corrected by solvent subtraction.

Thermodynamic Analysis. Folding has been considered in terms of a four-state model involving an equilibrium between the fully unfolded state, the two component β-hairpins, and the three-stranded antiparallel β-sheet. The observation that the two turns of **3**β show quite different temperature-dependent stability profiles indicates that a simple two-state folding model for **3**β is an oversimplification. We further justify the four-state model below. Each individual β-hairpin is subsequently analyzed in terms of a two-state folding model; the basis for this has been discussed elsewhere.[17] The equilibrium constant for the folding of each hairpin is given by $K = f_F/(1 - f_F)$, where f_F is the fraction of folded hairpin assessed using the chemical shift difference between the two Hα resonances ($\Delta\delta^{Gly}$) of either Gly9 or Gly17 in the two type I′ NG β-turns. Previously, we have used an RMS value of the deviation of all Hα chemical shifts from random coil values as a single parameter that provides a measure of the degree of folding at a particular temperature. This approach and the use of the Gly splitting data ($\Delta\delta^{Gly}$ values) for the turn residues used here have been shown to be in good agreement when deriving thermodynamic parameters from the temperature-dependence of the folded population.[19] The consistency between the two methods shows that the two-state approximation, in the context of hairpin folding, is a valid approximation since β-turn and β-strand residues appear to reflect the same folded population. $\Delta G°$ for folding was estimated from $\Delta G° = -RT \ln K$. The temperature-dependence of $\Delta\delta^{Gly}$ was fitted to the following expression, where $\Delta\delta^{Gly}_{limit}$ is the limiting value for the fully folded state:

$$\Delta\delta^{Gly} = \Delta\delta^{Gly}_{limit}[\exp(x/RT)]/[1 + \exp(x/RT)] \quad (1)$$

where

$$x = [T(\Delta S°_{298} + \Delta C_p° \ln(T/298)) - (\Delta H°_{298} + \Delta C_p°(T - 298))] \quad (2)$$

Initially, eq 1 was used iteratively to determine $\Delta H°_{298}$, $\Delta S°_{298}$, and $\Delta C_p°$ as $\Delta\delta^{Gly}$ varied with T. The limiting value for $\Delta\delta^{Gly}$ was determined from temperature-dependent data for peptide **2**β in 50% aqueous methanol where data were fitted assuming that $\Delta C_p°$ for folding is ∼0. This assumption is justified on the basis of the observation of a linear plot of $\Delta G°$ versus T for **2**β (X = Lys) in 50% methanol solution,[16] and from calorimetric studies of the unfolding of bovine ubiquitin at similar cosolvent concentrations where $\Delta C_p° \approx 0$.[18] The same limiting value for $\Delta\delta^{Gly}$ was assumed for Gly17 in the two peptides. When considering differences in stability ($\Delta\Delta G°$ values) between **2**β and **3**β, any error in this limiting value should be largely negated. While $\Delta G°$ values were determined directly from accurate chemical shift data, errors derived from the fitting procedure for $\Delta H°$, $\Delta S°$, and particularly $\Delta C_p°$ are expected to be significantly larger, as discussed previously.[19] Estimates of $\Delta C_p°$ for several analogues of **2**β at several pHs suggest that there is sufficient uncertainty in this parameter (±40%) as to preclude detailed comparisons between different hairpin analogues.[19] For this reason we do not attempt to overinterpret differences in $\Delta C_p°$ data presented in Table 1, other than to draw the conclusion that the data are consistent with a significant contribution from the hydrophobic effect to hairpin/β-sheet folding.[16] Thermodynamic data are presented in Table 1.

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Table 1. Thermodynamic Parameters (298 K) for the Folding of the β-Hairpin Components of the Three-Stranded Antiparallel β-Sheet Peptide 3β for Comparison with the Isolated C-Terminal Hairpin **2**β[a]

	$\Delta H°$ (kJ mol^{-1})	$\Delta S°$ (J K^{-1} mol^{-1})	$\Delta C_p°$ (J K^{-1} mol^{-1})
G9 (**3**β)	−17.0(±2.0)	−60.1(±5.0)	−860(±90)
G17 (**3**β)	1.4(±0.8)	0.9(±2.2)	−1400(±130)
G17 (**2**β)	−0.2(±0.5)	−6.1(±1.5)	−650(±80)

[a] All parameters are derived from the temperature-dependent splitting of the Hα resonances of the Gly residues ($\Delta\delta^{Gly}$ in Hz) in the NG turns; errors indicated are fitting errors. Uncertainties in $\Delta H°$ and $\Delta S°$ have been discussed in detail previously,[19] with the largest errors for $\Delta C_p°$ of up to ~40%. More realistic errors in $\Delta H°$ and $\Delta S°$ have been estimated on the basis of the range of values determined here and previously[19] using different independent NMR probes. We estimate a mean value for $\Delta H°$ of **2**β of −0.3(±1.3) kJ mol^{-1}, and for $\Delta S°$ a mean value of −9(±3) J K^{-1} mol^{-1}; errors for **3**β should be comparable.

CD analysis of the folding/unfolding equilibrium was followed by methanol titration assuming that the free energy of folding is linearly proportional to methanol concentration according to:

$$\Delta G_{fold} = \Delta G_{water} - m[\mathrm{MeOH}] \quad (3)$$

where ΔG_{fold} is the free energy of folding in aqueous methanol, ΔG_{water} the free energy of folding in water alone, m a constant of proportionality, and [MeOH] the concentration of methanol. The equilibrium constant for folding $K_F = f_F/(1 - f_F)$, where f_F is the fraction folded, was estimated from the experimental ellipticity monitored at 200 and 217 nm, assuming that $f_F = (\theta - \theta_U)/(\theta_F - \theta_U)$, where θ is the experimentally measured ellipticity, θ_U the ellipticity of the fully unfolded state, and θ_F the limiting value for the folded state. Thus, θ at a given concentration of methanol is related to ΔG_{water}, m, [MeOH], θ_U, and θ_F by the expression:

$$\theta = \{\theta_U + \theta_F \exp(-(\Delta G_{water} - m[\mathrm{MeOH}])/RT)\}/ \{1 + \exp(-(\Delta G_{water} - m[\mathrm{MeOH}])/RT)\} \quad (4)$$

ΔG_{water}, m, θ_U, and θ_F were determined iteratively from a nonlinear least-squares analysis using Kaleidagraph software (Synergy, Inc.). No assumptions were made with regard to precise limiting values for the fully folded or unfolded states. A number of different initial values for all iterated variables were used in the case of θ_{200}, but all converged to similar final parameters. There is more uncertainty in fitting the θ_{217} data because of the relatively small change in intensity at this wavelength. While the limiting value for the folded state θ_F is reasonably well determined by the experimental data at both wavelengths, the limiting value for the unfolded state θ_U is not, and was determined iteratively in the first instance. We examined the effects on ΔG_{water} of fixing θ_U at slightly different values to that determined iteratively, and estimated possible uncertainties in ΔG_{water} from the fitting analysis to be ±1 kJ mol^{-1}.

Structural Calculations. Starting structures for **3**β were randomly generated using DYANA version 1.5.[20] A total of 320 upper distance restraints derived from NOE data were classified as strong (<2.7 Å), medium (<3.8 Å), or weak (<5.0 Å). Restraints were checked for impact on structure using the "distance check" function which showed that there were no "lonely" NOEs that were unduly influencing the final conformation. Fifty structures were annealed using 4000 dynamics steps and 1000 minimization steps. Four structures chosen at random from the ensemble were further refined using molecular dynamics simulations using the SANDER module of AMBER 4.1,[21] with the same set of distance restraints. The four structures were first energy minimized and then submitted to 300 ps of dynamics using the SHAKE algorithm to allow a step size of 2 fs to be employed, with starting velocities assigned from a Maxwellian distribution at 50 K. The temperature was raised from 0 to 1000 K over the first 20 ps and held at 1000 K for a further 20 ps. The system was cooled to 300 K over a period of 20 ps and then held constant for the remainder of the simulation. The NOE distance restraints were introduced over the first 2 ps by ramping the restraint force constant from 0 to 32 kcal·mol^{-1}. An implicit solvation model was used, employing a distance-dependent dielectric and an electrostatic cutoff of 9 Å. Ten structures, representing the fully folded state, were extracted from the final 100 ps of each trajectory. These were energy minimized and analyzed using WHATCHECK[22] and MOLMOL.[23] The sequence adopts a twisted β-sheet conformation with >90% of residues found to lie in favorable regions of Ramachandran space. No NOE distance restraints were violated by >0.4 Å, and no torsion angle restraints by >15°. The mean backbone RMSD within this family of 10 structures was 1.13 ± 0.22 Å which rises to 1.52 ± 0.23 Å when all heavy atoms are considered. Of the 12 possible cross-strand hydrogen bonds expected for 3β, only an average of 7 (60%) are formed per structure.

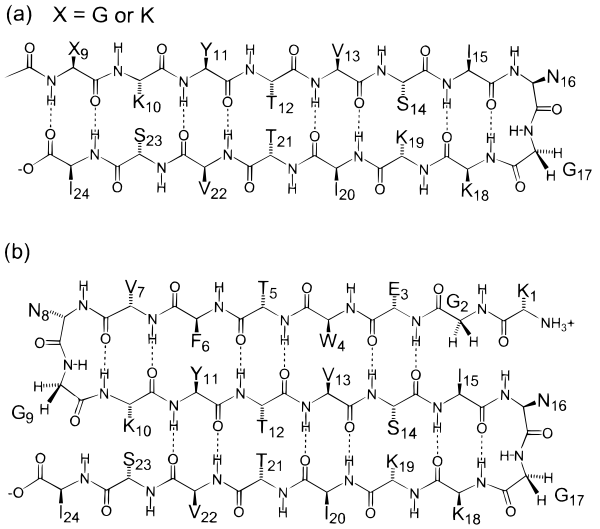

Figure 2. Amino acid sequences (one letter code) for peptides (a) **2**β and (b) **3**β with backbone alignment and interstrand hydrogen bonding interactions indicated; the amino acid numbering system used for both peptides is derived from **3**β (1−24) with the C-terminal hairpin **2**β defined as residues 9−24.

Results and Discussion

Cooperative Interactions Parallel to the Strand Direction. Previously we have shown that peptide **2**β (Figure 2) folds in aqueous solution to form a significantly populated β-hairpin.[16,17] The stability of **2**β (X = Lys) has a marked pH-dependence which we have attributed to a salt bridge between Lys9, Lys10, and the C-terminal carboxylate group of Ile24.[19] At low pH, these interactions are turned off by neutralizing one of the charges ($CO_2^- \rightarrow CO_2H$), resulting in a change in the population of the folded state. Notably, all Hα chemical shifts of the peptide are perturbed by the change in pH, including the distant turn residues Asn16 and Gly17, establishing that localized changes in interactions between the N- and C-termini are propagated throughout the β-hairpin structure, demonstrating a significant degree of cooperative stabilization parallel to the β-strand direction (Figure 1a). Here we examine quantitatively the cooperative effects of an additional β-strand on hairpin stability by adding further weak interactions in a direction orthogonal to the β-strands (Figure 1b).

Design of a Three-Stranded Antiparallel β-Sheet. We have extended peptide **2**β (X = Gly) with an additional type I′ Asn-

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Gly turn and a complementary third strand (Figure 2) that utilizes a cluster of aromatic residues as an interstrand stabilizing motif. We introduced Phe and Trp in non-hydrogen bonding sites on the third strand to complement Tyr and Val residues on the opposing strand (Figure 2b). Such a quartet on one face of the sheet buries a significant hydrophobic surface area but also allows favorable edge−face and offset $\pi-\pi$ interactions to add stability. A similar, but not identical, motif is found in the B1 domain of protein G;[24] the stability of an isolated β-hairpin derived from this native structure has also been attributed to the interactions of these residues.[25] A more detailed thermodynamic characterization has been reported for this peptide,[26] together with a model hairpin with a similar motif of aromatic residues.[27] The N-terminal residues of our designed peptide (EGK) were added in part to enhance solubility. To eliminate steric interactions between the bulky indole ring of Trp4 and its $i + 2$ neighbor, a Gly residue was placed at the $i + 2$ position (*WEG*), permitting the indole ring to pack favorably against the second strand rather than forming competing intrastrand interactions.

Circular Dichroism Analysis of the Folding of 3β. Far-UV CD spectra of **3**β were recorded in water and at various concentrations of methanol at 293 K (Figure 3). In water, the CD spectrum shows pronounced negative ellipticity at 198 and 217 nm, indicative of the presence of β-sheet structure in equilibrium with random coil.[28] Titration with methanol solution at a fixed concentration of peptide shows a marked increase in the negative ellipticity at 217 nm with θ becoming positive below ~202 nm. As shown in previous studies,[29] the effects of the cosolvent appear to be to perturb the folded ↔ unfolded equilibrium toward the folded state enhancing the intrinsic conformational propensity of the peptide.

By analogy with previous methods of estimating the α-helical content of peptides, based on the assumption of a linear dependence of the free energy change for folding on methanol concentration (eq 3 in Methods),[30] we have estimated the average β-sheet content of **3**β using a similar approach. In applying this model to helix stability, Sancho et al.[30a] have emphasized that the linear extrapolation approach applies equally well to multi-state equilibria, in which the folded state is an ensemble of conformational states. In Figure 3b, mean residue ellipticity (θ) is plotted against methanol concentration (%) at two wavelengths, 200 and 217 nm, and the line of best fit to eq 4 shown (as described in Methods). The linear free energy relationship seems to be a reasonable approximation when applied to this data set. Thus, we estimate ΔG_{water} for the folding of **3**β to be $+0.02(\pm1)$ kJ mol^{-1} at 293 K indicating that **3**β is

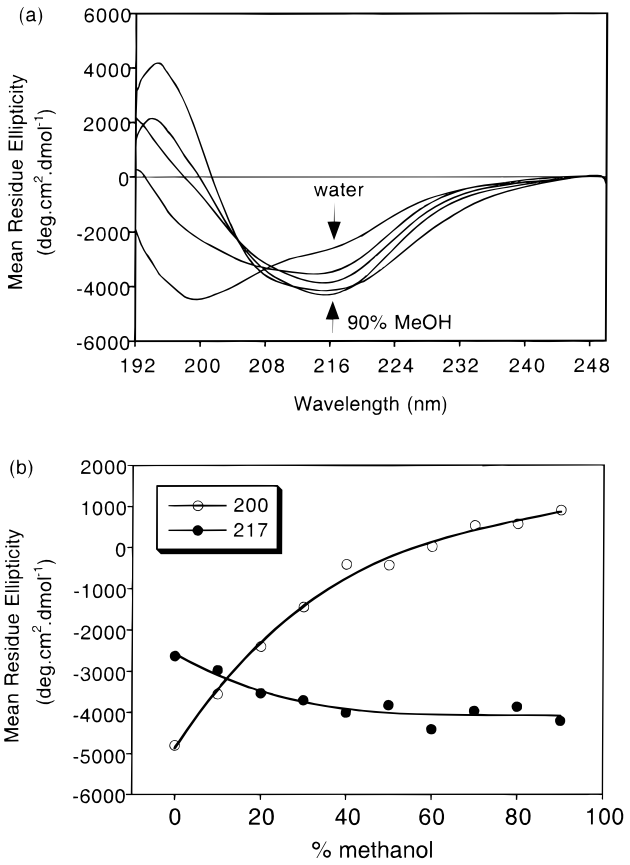

Figure 3. (a) Far-UV CD spectra (190−250 nm) of peptide **3**β recorded as a function of methanol concentration at a fixed peptide concentration of 50 μM at 293K; (b) plot of mean residue ellipticity at 200 and 217 nm as a function of methanol concentration; the line of best fit to eq 4 is shown in each case.

approximately 50(±10)% folded under these conditions (see Methods). We interpret this as indicating that on average each residue is occupying β-space for approximately 50% of the time averaged over the entire conformational ensemble, rather than 50% of the molecules being folded into a regular three-stranded antiparallel β-sheet and 50% fully random coil, as would be required for a two-state folding model. As we discuss and justify below, the four-state model appears to be a more appropriate approximation, and consistent with the NMR data.

NMR Analysis of Folding of 3β. NMR studies of **3**β in aqueous solution indicate that both type I′ NG turns (Asn8-Gly9 and Asn16-Gly17) are highly populated, showing large deviations of Hα shifts from random coil values (Figure 4a).[31] The spectra are well-resolved, as illustrated in the overlayed NH-Hα regions of the NOESY and TOCSY spectra shown in Figure 4b, where sequential connectivities between residues are highlighted. Residues in the β-strands of **3**β generally show significant downfield shifts consistent with the pattern expected for a three-stranded antiparallel β-sheet punctuated with two β-turns,[32] although residues in the N-terminal strand are likely to be perturbed by ring current effects from W4 and F6. The proposed strand alignment and register of interstrand hydrogen bonds is confirmed by detailed NOE studies which identify many long-range main chain−main chain interactions involving Hα and NH protons (Hα-Hα: W4→V13, F6→Y11, T12→T21,

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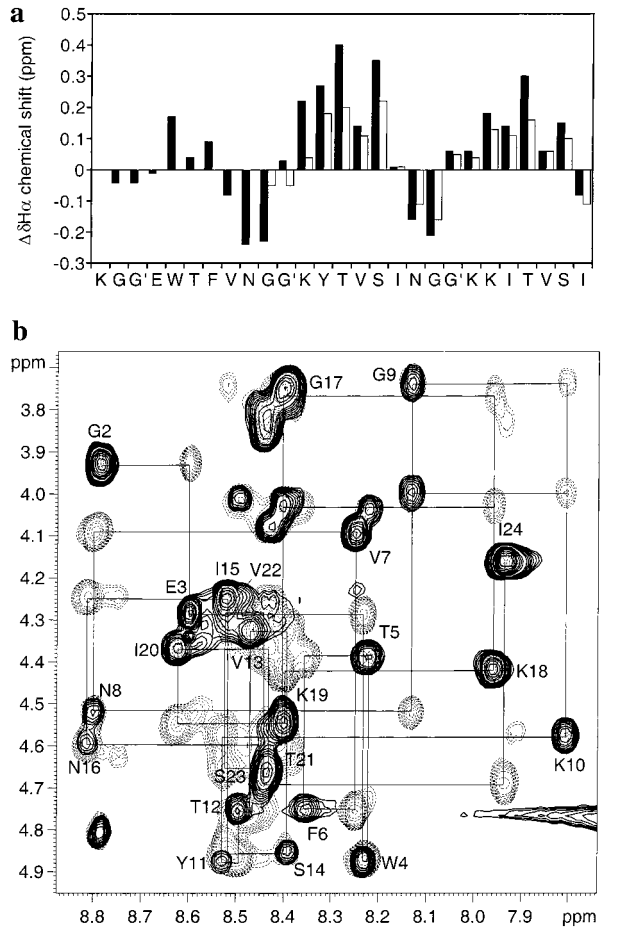

Figure 4. (a) Deviations of Hα chemical shifts from random coil values ($\Delta\delta^{H\alpha}$) at 298 K for **2**β (open bars) and **3**β (black bars) in aqueous solution; (b) overlayed portions of the H_2O NOESY and TOCSY spectra of **3**β recorded at 298 K showing NH-Hα correlations along the peptide backbone.

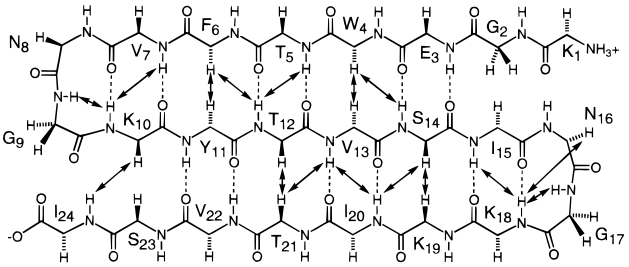

Figure 5. Observed interstrand NOEs involving backbone Hα and NH that are consistent with the proposed interstrand alignment and pattern of hydrogen bonds (data collected at 298 K).

and S14→K19; and NH−NH: V7→K10, T5→T12, I15→K18, and V13→I20; summarized in Figure 5). In addition, we see a number of side chain−side chain NOEs between W4, F6, and Y11 that define their relative orientation on the same face of the β-sheet, and which are consistent with a stabilizing contribution through $\pi-\pi$ interactions. Many other interstrand hydrophobic contacts between aliphatic residues are also evident from the NOE data (not shown).

On the basis of 320 NOE distance restraints we have calculated a family of structures of the fully folded state consistent with the experimental data, using a combination of torsion angle driven dynamics (DYANA)[20] and molecular dynamics simulations (AMBER 4.1).[21] A family of 40 structures, together with a single average structure is illustrated in Figure 6. The overall topology of **3**β is evident, together with the relative position and orientation of aromatic and hydrophobic interactions on one face of the twisted β-sheet. Both NG turns adopt the two-residue type I′ conformation, giving the structure a pronounced twist, typical of protein β-sheets. The aromatic side chains are observed to interact in energetically favorable edge−face orientations. Of the possible 12 cross-strand hydrogen bonds involving the three strands, only an average of 60% of these are formed per structure.

Evidence for Cooperative Interactions between β-Strands. Hα chemical shifts have been used widely as a measure of the extent of folding[14,32,33] since downfield shifts for residues in β-strands appear to reflect largely the proximity of an Hα to a carbonyl group on the opposing strand. In Figure 4, we compare Hα deviations from random coil shifts[31] ($\Delta\delta^{H\alpha}$) for **3**β with those for **2**β (X = Gly) under identical conditions. It is evident that many residues common to both peptides (see, for example, Thr12 and Thr21) show larger $\Delta\delta^{H\alpha}$ values in the three-stranded sheet, reflecting an increased population of the β-hairpin structure in the presence of the third strand. In the folded conformation the Gly residue in the NG β-turn experiences a highly anisotropic environment such that each Hα resonance has a quite different chemical shift. This chemical shift difference ($\Delta\delta^{Gly}$) is particularly sensitive to the folded population[19] and has been used as a convenient measure of the relative stabilities of the hairpin in the presence and absence of the third strand (see Methods). We have assumed, as a first approximation, a four-state model for folding involving an equilibrium between the fully unfolded state, the two component β-hairpins, and the three-stranded antiparallel β-sheet (Figure 7). Each individual β-hairpin is analyzed in terms of a two-state folding model, as previously discussed (see Methods).[17] We have determined $\Delta G°$ for folding for each hairpin as a function of temperature from the $\Delta\delta^{Gly}$ data (Figure 8).[34,35] It is evident from the data for Gly17 that the NG turn of **3**β is more highly populated than the corresponding turn of the isolated hairpin,

(33) Lacroix, E.; Kortemme, T.; De La Paz, M. L.; Serrano, L. *Curr. Opin. Struct. Biol.* **1999**, *9*, 487.

(34) We justify a four-state model on the basis that **2**β has already been shown to fold autonomously in solution, while the work of others has established that β-hairpins with a motif of aromatic residues, analogous to the N-terminal hairpin of **3**β, also fold autonomously in water with a similar stability, and via an approximate two-state mechanism.[26,27] Although we report here the use of $\Delta\delta^{Gly}$ values from the Hα splitting of the Gly residue in the turn as a measure of stability, we have previously shown[19] that Hα shifts of residues in the β-strands show the same temperature-dependence, indicating that all residues reflect the same folded population. We have used this evidence to support the two-state model for folding of hairpin **2**β; a similar approach has been used by others.[26,27] We have applied the same methods to the characterization of **3**β**.** We can show that Hα shifts for β-strand residues in the N-terminal and C-terminal hairpins of **3**β give similar stability profiles to those for the turn Gly residues. We have calculated a RMS deviation of Hα shifts from random coil values (RMS ΔδHα) for inward pointing Hαs in the β-strands, as previously described,[16,17,19] and find a very similar temperature-dependent stability profile to those seen in Figure 8 using $\Delta\delta^{Gly}$ values, with the N- and C-terminal hairpins again showing different characteristics. The data support an approximate two-state folding of the individual hairpins of **3**β. The relatively weak cooperativity between strands, together with the similar overall stabilities observed for the two constituent hairpins, as well as the fully folded **3**β and the random coil conformation, suggests that at equilibrium all of these conformational states will be significantly populated (see the equilibria in Figure 7), and that a four-state model is an appropriate approximation.

(35) We conclude that the requirements for a three-state model, with one hairpin significantly folded and the other unfolded, with a marked degree of cooperativity between the folded hairpin and the third strand, are not met by the experimental data. As shown by the stability profiles in Figure 8, according to the experimental conditions, the C-terminal hairpin is of greater stability at high temperature (320 K), while the N-terminal hairpin is more highly populated at lower temperature (280 K), with equal populations at 299 K. However, no single hairpin conformation dominates at any particular temperature.

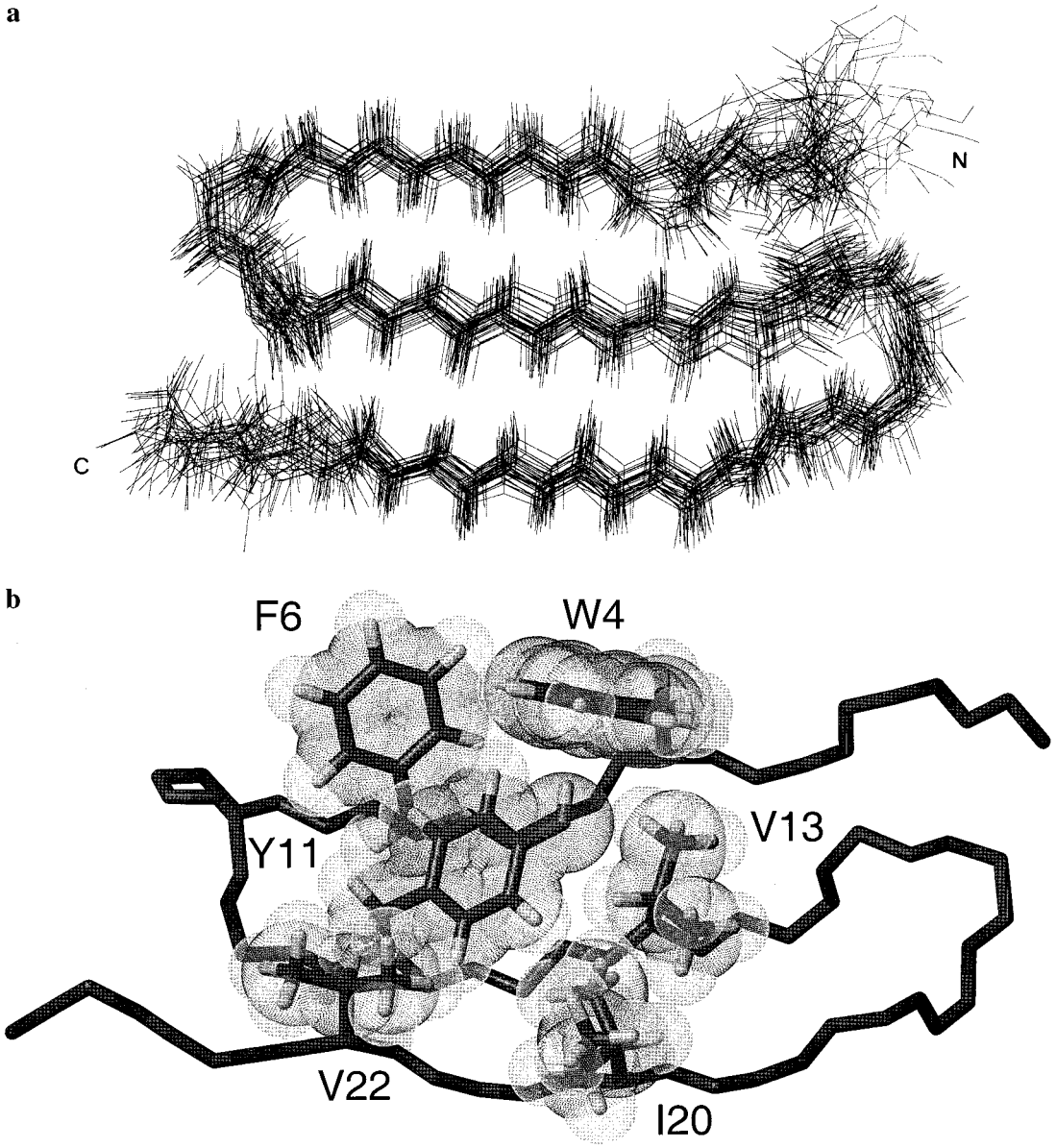

Figure 6. (a) Family of 40 NMR structures showing the fold of the peptide backbone of **3***β*, based on NOE restraints from data at 298 K; (b) single average structure showing main chain alignment and the position and orientation of interacting aromatic residues and hydrophobic contacts on one face of the *β*-sheet.

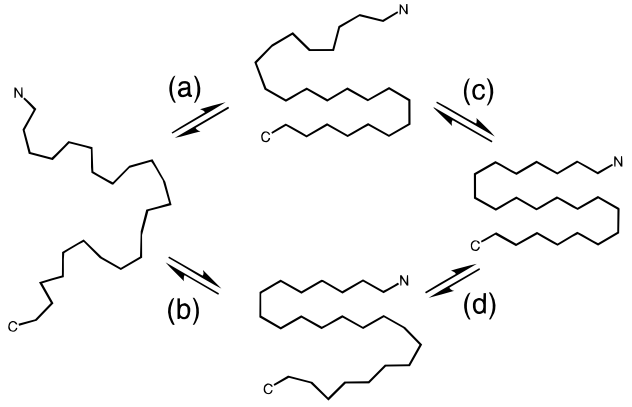

Figure 7. The four-state model for peptide folding with the unfolded state in equilibrium with the two component *β*-hairpins and the three-stranded antiparallel *β*-sheet: (a) folding of C-terminal hairpin, (b) folding of N-terminal hairpin, (c) folding of N-terminal hairpin to **3***β*, and (d) folding of C-terminal hairpin to **3***β*. Folding of each hairpin [(a) and (b)] is considered to approximate to a two-state process.

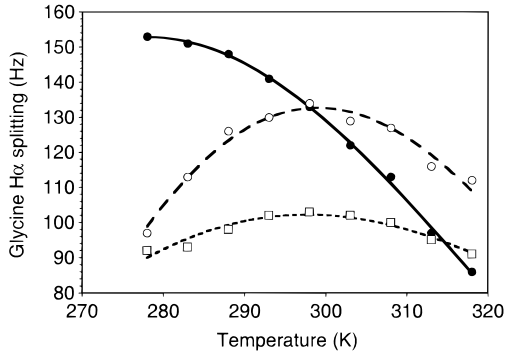

Figure 8. Temperature-dependence of Gly Hα splitting ($\Delta\delta^{Gly}$ in Hz) of the *β*-turn residue G17 in **2***β* (open squares) and **3***β* (open circles) and from G9 in **3***β* (solid line); lines of best fit to eq 1 are shown, with thermodynamic parameters presented in Table 1.

2*β*. This is also reflected in differences in Hα chemical shifts for other residues common to both peptides. We estimate a difference in stability for **2***β*, in the presence and absence of

the third strand, of 1.1 kJ mol^{-1} at 298 K, reflecting an increase in folded population from 30% to 40%.

Noticeably, $\Delta G°$ for folding shows a marked nonlinear temperature-dependence that results in unfolding of the C-terminal hairpin of **3**β at low temperature. Fitting the data shows that folding is associated with a significant negative $\Delta C_p°$, with enthalpy and entropy terms close to zero. These observations are consistent with hydrophobic stabilization in water through burial of aliphatic side chains at the interface between the two β-strands.[16,17] We have performed a similar thermodynamic analysis for the second turn of **3**β using the Gly9 data to compare the stability of the two hairpin components. The temperature−stability profile is quite different, showing less pronounced curvature and the absence of the cold denaturation seen for the C-terminal hairpin. The temperature-dependence of $\Delta\delta^{Gly}$ is shown in Figure 8 for **2**β and **3**β. In this case, folding of the N-terminal β-hairpin of **3**β is strongly enthalpy-driven with a negative entropy change (Table 1). This thermodynamic signature would appear to be consistent with our design principle that $\pi-\pi$ interactions should be stabilizing in the N-terminal hairpin of **3**β, and that these interactions should have an electrostatic (enthalpic) origin.[36] Studies of a β-hairpin peptide derived from the B1 domain of protein G, which carries a similar motif of aromatic residues, have also been shown to give an analogous thermodynamic signature indicative of enthalpy-driven folding,[25b] but the authors neglected to take into account the effects of $\Delta C_p°$. More recent analyses of the same native hairpin,[26] and a designed hairpin with an analogous aromatic motif,[27] have identified similar thermodynamic signatures.

The different temperature-dependent stability profiles of the two turns of **3**β show that the peptide does not fold to a three-stranded antiparallel β-sheet in a single cooperative event, for which we would expect the two turns to exhibit identical thermodynamic profiles. Rather, we have the unique situation in which the N-terminal β-hairpin becomes more folded at low temperature, while the C-terminal hairpin becomes less folded. Thus, a two-state model is a rather simplistic description of events, a fact that is readily apparent in this context. While folding is not a highly cooperative two-state process, the type I′ turn of **2**β shows a small but significant increase in stability in the presence of the third β-strand demonstrating cooperative interactions between strands. Our observations are in good agreement with the qualitative results of Schenck and Gellman,[10a] who used a ^{D}Pro-Gly to ^{L}Pro-Gly turn-mutation to switch "on" and "off" the interaction of the third strand. In contrast, studies by De Alba et al.[11] of the folding of a three-stranded antiparallel β-sheet could find little evidence for cooperative interactions between strands.

Conclusions

In a *de novo* designed three-stranded antiparallel β-sheet (**3**β) we have demonstrated cooperative interactions between β-strands. Although the origin of this effect is still uncertain, pre-organization of one or another β-hairpin seems likely to enhance β-sheet formation on entropic grounds by templating the docking of a third β-strand.[7,8] We have presented a four-state model for folding in which both the N- and C-terminal β-hairpin conformations are significantly populated, both providing possible templates for interaction with the third strand. In such a small peptide system, the extent of backbone pre-organization is unlikely to compare with that in a native protein β-sheet. More likely, hydrophobic contacts between side chains stabilize a collapsed conformation[37] where interstrand hydrogen bonding may play a relatively minor stabilizing role.[15,38] This may explain why cooperative interactions appear to have such a small effect on overall stability, because the observed effects are mediated by relatively "loosely" defined interactions between side chains rather than a regular, extended "crystalline" network of hydrogen bonds.[39] Although the two constituent β-hairpins of **3**β show roughly similar stabilities at 298 K, we have demonstrated that they have quite different thermodynamic profiles, reflecting the nature of the side chain−side chain interactions stabilizing the various component β-strands. Both enthalpic and entropic contributions to the folding of **2**β are very close to zero which, together with a significant negative $\Delta C_p°$ for folding, are consistent with the classical hydrophobic stabilization model expected for interactions involving aliphatic residue side chains.[40] Introducing a motif of aromatic interactions within the N-terminal hairpin structure produces an enthalpy-driven interaction between strands,[26,27] consistent with $\pi-\pi$ interactions being electrostatic in origin.[36] The use of such a cluster of residues, related to that of a native protein motif,[24] together with the Asn-Gly sequence, which has a high propensity for type I′ turn formation,[17,41] has proved successful in the *de novo* design of a three-stranded β-sheet peptide that folds to its target structure.

The results presented here for a simple peptide system demonstrate a contribution to β-sheet propagation and stabilization through an incremental effect involving cooperative interactions perpendicular to the strand direction. In the context of nucleation−condensation mechanisms for protein folding,[42] the formation of a transition state, and subsequent commitment to a particular folded topology, may depend on a network of stabilizing interactions and the weakly cooperative interplay between them that takes place in the final rapid folding events.[43] This simple model system provides some insight into the weakly cooperative interactions relevant to these events.

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