The relative positions of alanine residues in the hydrophobic core control the formation of two-stranded or four-stranded α-helical coiled-coils

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The objective of this study was to investigate the positional effect of hydrophobic interactions in the α-helical interface in controlling the formation of two-stranded and four-stranded coiled-coils. Two disulfide-bridged antiparallel coiled-coils were designed which differ only in the position of a single Ala residue in the middle heptad: in peptide 2H the Ala residues are in register (in the same rung), while in peptide 4H they are not. Data from size-exclusion chromatography and sedimentation equilibrium experiments showed that under benign conditions peptides 2H and 4H were two-stranded and four-stranded coiled-coils respectively. These results, in conjunction with molecular modeling studies, suggest that when four Ala residues are in the same plane of a potential four-stranded coiled-coil, the small side chains of Ala would create a large cavity in the hydrophobic interface of the potential four-stranded structure which is destabilizing and favors the two-stranded, disulfide-bridged coiled-coil. In contrast, an alternating Leu-Ala hydrophobic packing in the two planes distributes the potential cavity over a larger region, which may be partially filled by minor adjustments of the neighboring Leu side chains. As a result, there is still sufficient hydrophobic contact to maintain the four-stranded structure.

Keywords: α-helices/hydrophobic packing/multiple helix coiled-coil/protein folding

Introduction

The naturally occurring forms of α-helices can be classified into three broad categories. The first group consists of α-helices that are part of the secondary structure of globular proteins. The second group includes the coiled-coils, which may be two-stranded (Cohen and Parry, 1990; Conway and Parry, 1990; Hodges, 1992; Adamson et al., 1993), three-stranded (Wilson et al., 1981; Sorger and Nelson, 1989; Weis et al., 1990; Conway and Parry, 1991; Peteranderl and Nelson, 1992; Lovejoy et al., 1993) or four-stranded coiled-coils (Weber and Salemme, 1980; Banner et al., 1987; DeGrado et al., 1989; Paliakasis and Kokkinidis, 1992). In the latter group the amino acid sequence contains the 3,4-hydrophobic repeat that is characteristic of coiled-coils. The α-helical chains are oriented parallel and/or antiparallel to each other and are stabilized by specific packing of hydrophobic residues in the a and d positions of the heptad repeat, where the amino acid residues are denoted by the letters abcd/ef. The third group is made up of multiple-helix bundles, where each chain is α-helical but the hydrophobic interface is more complex than the narrow hydrophobic face of the 3,4 repeat of coiled-coils. Both the multiple-helix coiled-coils and bundles are stabilized by electrostatic and hydrophobic interactions and the terms multiple-helix 'bundles' and 'coiled-coils' appear to be used interchangeably in the literature. For consistency, we are using the term multiple-helix 'coiled-coil' when the hydrophobic packing in the interface involves mainly 3,4-hydrophobic repeats. The multiple-helix bundle contains wide variations in both the packing of hydrophobic residues in the interface and the relative orientations of the α-helical chains with respect to each other. Examples of the third group include mellitin (Terwilliger and Eisenberg, 1982; Terwilliger et al., 1982), myohemerythrin, cytochrome c, influenza virus hemagglutinin HA2 (Richardson, 1981) and some of the cytokines (Mott and Campbell, 1995).

Despite many attempts to identify the critical structural elements that control the formation of multiple-stranded coiled-coils (DeGrado et al., 1989; Cohen and Parry, 1990), it is still not known what interactions determine the specificity of the state of association or 'multiple-strandedness' of coiled-coils. To illustrate this point, a peptide sequence that was originally designed to form a two-stranded parallel coiled-coil (O'Neil and DeGrado, 1990) was later identified from X-ray crystallographic data as a triple-helix coiled-coil (Lovejoy et al., 1993). A synthetic peptide with Ile in all five d positions and Leu in all five a positions was a four-stranded coiled-coil (Alber, 1992), while a similar peptide with only three Ile residues in the middle d positions was two-stranded (Zhu et al., 1993). In addition, a 35-residue peptide with Leu at all the a and d positions was two-stranded in the reduced form, but the disulfide-bridged coiled-coil became four-stranded (Zhu et al., 1993). Analogs of the Rop protein, where the hydrophobes in the interface were exclusively replaced with alternating Leu-Ala residues, appeared to exhibit the native-like four-stranded structure (Munson et al., 1994). Finally, variations in the hydrophobic interface of a synthetic GCN4 peptide resulted in the formation of coiled-coils with two, three or four helices (Harbury et al., 1993). In GCN4, it was shown that an homogeneous isoleucine interface (Ile at a and d positions) resulted in a trimeric coiled-coil (Harbury et al., 1994) and substitution of leucine into the a positions in this trimeric helix can favor tetramer formation (Harbury et al., 1993). A search of the literature will reveal many more examples of these 'subtle' differences in amino acid sequence that led to different states of association of α-helical chains in coiled-coil assemblies.

Hydrophobic interactions stabilize both two-stranded and four-stranded coiled-coils and it is reasonable to assume that the relative stabilities of the two conformations determine the preferred structure in solution. It has been estimated that more surface area per helix is buried in the four-stranded than in the two-stranded coiled-coil (Harbury et al., 1993), yet many naturally occurring coiled-coils are also two-stranded. Therefore, the aim of this study is to investigate how specific
packing of Leu and Ala residues in the α-helical interface controls the specificity of the formation of the two- and four-stranded coiled-coils. We used antiparallel coiled-coils because the orientation of α-helical chains in most naturally occurring four-helix coiled-coils is such that the neighboring helices are antiparallel to each other (Banner et al., 1987; DeGrado et al., 1989; Cohen and Parry, 1990).

Materials and methods

Peptide synthesis and purification

The starting peptides were synthesized using solid-phase methodology, purified by reversed-phase HPLC and their identities were confirmed by amino acid analysis and by mass spectrometry as described previously (Monera et al., 1993).

Air oxidation and purification

Air oxidation for disulfide bridge formation was accomplished by dissolving a mixture of 5 mg each of the reduced peptides in 5 ml of 100 mM NH₄HCO₃, pH 8.3, and magnetically stirring the solution in an open vial at 20°C. The progress of oxidation was monitored by reversed-phase chromatography using 50 μl aliquots of the reaction mixture. The instrumentation consisted of a Hewlett-Packard HP-1090 liquid chromatograph equipped with an analytical C8 column (Zorbax 300SB-C8, 4.6 mm × 25 cm, 5 μm particle size, 300 Å pore size). The oxidation products were eluted at 1 ml/min using a linear AB solvent gradient of 2% B/min, where solvent A was 0.05% TFA in water and solvent B was 0.05% TFA in acetonitrile. Oxidations were typically complete in 15-24 h.

To separate and purify the oxidized products, the peptide solutions were neutralized with dilute acetic acid and then injected into the Varian Series 5000 liquid chromatograph equipped with a semi-preparative reversed-phase C8 column (Zorbax 300SB-C8, 9.4 mm i.d. × 25 cm, 5 μm particle size, 300 Å pore size). The samples were eluted at 2 ml/min with a linear AB gradient of 1% B/min for the first 15 min and 0.2% B/min thereafter, where solvent A was 0.05% TFA in water and solvent B was 0.05% TFA in acetonitrile. Aliquots of the fractions were subjected to analytical HPLC and pure fractions were pooled and lyophilized. In all cases, the identities of the products were confirmed by amino acid analysis, mass spectrometry and reduction and HPLC characterization of the resulting reduced peptides.

Circular dichroism spectroscopy

Stock solutions of disulfide-bridged peptides (≈10 mg/ml) were prepared by dissolving 3 mg of peptide in 300 μl of benign buffer (50 mM phosphate containing 0.1 M KCl, pH 7). Two sets of peptide solutions were prepared for CD studies: one was in benign buffer (10 μl stock peptide solution was diluted with 20 μl of the same buffer and 30 μl water) and the other was in 50% TFE (10 μl stock peptide solution was diluted with 20 μl buffer and 30 μl TFE). The peptide solutions were then loaded into an 0.02 cm fused silica cell and their ellipticities were scanned from 190 to 250 nm.

Circular dichroism spectroscopy was performed at 20°C on a Jasco J-500C spectropolarimeter (Jasco, Easton, MD) equipped with a Jasco IF-500II interface connected to an IBM PS/2 model 30286 computer using Jasco DP-500/PS2 system, version 1.33a software. A Lauda water bath (Model RMS; Brinkmann Instruments, Rexdale, Ontario, Canada) was used to control the temperature of the cell. The instrument was calibrated daily with an aqueous solution of recrystallized ammonium-d-10-(+)-camphorsulfonic acid at 290.5 nm. The ellipticity at 220 nm is reported as the mean residue ellipticity ([θ], deg. cm² dmol⁻¹) and calculated from the equation [θ] = [θ]₀ₑᵤ(mrnw)/10lc, where [θ]₀ₑᵤ is the molar ellipticity measured in degrees, mrnw is the mean residue weight (molecular weight of the peptide divided by the number of amino acid residues), c is the peptide concentration in g/l and l is the optical path length of the cell in cm. Four CD scans from 250 to 190 nm were collected at 0.25 nm intervals and averaged to give the final CD spectra. The standard deviation of measurements at 220 nm was ±300 deg. cm² mol⁻¹.

Denaturation studies

A 10 M urea stock solution was prepared in 50 mM phosphate, pH 7 and aliquots were used to prepare a series of solutions containing different concentrations of urea. The ellipticities of the peptides at different denaturant concentrations were recorded at 220 nm from the average of eight readings. The fraction of peptide in the folded state ([θ]ₚ) was then calculated from the equation [θ]ₚ = ([θ]₀ₑᵤ - [θ]₀ₑᵤ(α))/([θ]₀ₑᵤ - [θ]₀ₑᵤ(β)), where [θ]₀ₑᵤ(α) is the observed mean residue ellipticity at any denaturant concentration and [θ]₀ₑᵤ(α) and [θ]₀ₑᵤ(β) are the mean residue ellipticities for the native (folded) and unfolded states respectively. Thus, the fraction of peptide in the unfolded state is represented by the relationship [θ]ₚ = 1 - [θ]ₚ. Peptide 2H was analyzed on the basis of a monomolecular unfolding process, F <-> U, where the equilibrium constant is Kᵤ = [U]/[F]. This assumption is based on the existence of monomeric species from size-exclusion chromatography and sedimentation equilibrium experiments, the presence of a single transition in three different denaturation experiments and the presence of an isodichroic point at 203 nm in the temperature denaturation wavelength scans, all of which will be discussed in later sections. In contrast, peptide 4H was analyzed based on a bimolecular, two-state unfolding process, F <-> 2U. The equilibrium constant, Kᵤ, is then calculated from the equation Kᵤ = [U]/[F] = 2P_i/(fₚ/fₚ), where P_i is the total protein concentration. Similarly, the assumption that folded monomers do not exist in a measurable amount even after extended time periods is based on the results from size-exclusion chromatography and sedimentation equilibrium experiments showing the existence of only dimeric species (four-stranded), the presence of a single transition in three different denaturation experiments and the presence of an isodichroic point at 203 nm in the temperature denaturation wavelength scans. In both cases, the free energy of unfolding at each denaturant concentration was calculated from the equation ΔGᵤ = -RT ln Kᵤ and the free energy of unfolding in the absence of denaturant, ΔGᵤ[^H2O], was calculated by the linear extrapolation method (Pace, 1986; Santoro and Bolen, 1992), based on the equation ΔGᵤ = ΔGᵤ[^H2O] - m[urea], where [urea] is the urea concentration and m is the slope term.

Alternatively, assuming that the free energy of unfolding is a linear function of the denaturant concentration, the fractions of unfolded peptide 2H were fitted to the equation:

\[
[θ]ₚ = \frac{\exp(-(ΔGᵤ[^H2O] - m[urea])/RT)}{[1 + \exp(-(ΔGᵤ[^H2O] - m[urea])/RT)]}
\]

Similarly, the equation [θ]ₚ = k²/2Pₐ(1 + (1 + 8Pₐ/k²)²)², where k = \exp \left(-(ΔGᵤ[^H2O] - m[urea])/RT\right), was used in the case of peptide 4H. The values of m and ΔGᵤ were determined by non-linear least-square curve fitting, which are probably more accurate estimates because all data points are included.
Temperature denaturation

Each peptide solution (~50 μM peptide in 25 mM P04/50 mM KCl, pH 7) was loaded into an 0.02 cm cell and its ellipticity was scanned from 195 to 250 nm at different temperatures. The ellipticity readings at each temperature were converted into molar ellipticities and the fraction of peptide in the folded state was calculated as the fraction of the residual molar ellipticity at each temperature relative to the initial molar ellipticity at 5°C. This is based on the observation that the molar ellipticities of these peptides in 8 M urea is negligible (~±500 deg. cm² dmol⁻¹ at 220 nm). The melting temperature, t_m, is then taken as the temperature at which half of the peptide is unfolded.

Size exclusion chromatography

Approximately 1 mg of disulfide-bridged peptides were dissolved in 0.2 ml of 50 mM phosphate buffer containing 0.1 M KCl, pH 7. A 50 μl aliquot was injected into a Hewlett-Packard model HP 1090 liquid chromatograph equipped with a Superdex 75 size exclusion column (Pharmacia Fine Chemicals) and eluted with the same buffer at 0.5 ml/min. Size-exclusion chromatography was also performed under denaturation conditions by dissolving and running the peptides in the same buffer containing different concentrations of GdnHCl.

Sedimentation equilibrium ultracentrifugation

The peptides were dissolved in 50 mM P04 containing 1 M KCl, pH 7.0, to give 1.5 mg peptide per ml solution and then dialyzed for 48 h against the same buffer at 4°C. The peptide concentration was determined by fringe count using 4.1 fringes equivalent to 1 mg/ml (Babu and Stellwagen, 1969) and amino acid analysis. Sedimentation equilibrium experiments were carried out in a Model E analytical ultracentrifuge with an interference optical system at a speed of 26 000 r.p.m., as described in earlier publications (Kay et al., 1991; Zhu et al., 1993) and using a partial specific volume of 0.73 ml/g.

Molecular modeling

The structures of the peptides 2H and 4H were modeled as four-helix and two-helix coiled-coils. The models were generated using INSIGHTII, BIOPOLYMER and DISCOVER and the Consistent Valence Force Field (CVFF) (Biosym, San Diego, CA). The initial coordinates for the antiparallel four-stranded helical coiled-coil were obtained from the crystal structure of the Rop protein, pdb entrance pdblrrop.ent (Banner et al., 1987). The average backbone coordinates for single, individual coils were obtained by superposition and averaging of the individual chains in Rop and by superimposing and averaging the different heptad repeats of several copies of this average coil translated by one heptad to obtain an elongated (42 residues) single average coil built of identical heptad repeats. Subsequently, the amino acid residues were replaced to conform with the sequence of the regular, all-leucine parent peptide 4HL (Figure 1), that is five repeats of (KLEALEG) and (ELAELKG) with a Cys residue at positions 2 and 33 for chains 1 and 2 respectively. Side chain dihedral angles were obtained from a library of preferred rotamers (Ponder and Richards, 1987). Two copies of these two chains were then superimposed on the backbone of the Rop dimer generated from the crystal coordinates by symmetry operation to obtain the correct interhelical spacing and alignment. This operation inherently assumes an all-antiparallel alignment of the coils, which is justified since it is the only possible dimer orientation resulting in completely favorable charged side chain interactions. After disulfide bond formation between adjacent coils, this initial four-helical coiled-coil model was refined using a four-step energy minimization procedure. Each step consisted of 100 steps of steepest descent and 1000 steps of conjugate gradient minimization in vacuo using a distance-dependent dielectric constant of 4 and an additional harmonic potential enforcing peptide bond planarity. The steps differed by the decreasing use of fixing and tethering atom coordinates (using a force constant of 100 kcal/mol Å²) from the first step fixing all heavy atoms to the last step using no constraints on atom positions. This procedure was applied to several variants of the model, differing in the dihedral angles of the leucine residues in the core. After analysis of the resulting structures' conformational energies, dihedral angle changes, accessible surface areas and packing defects, clear differences in these models suggested the leucine rotamer with χ₁ = 180 and χ₂ = 180 and χ₁ = 180 and χ₂ = 60 in the a and d positions respectively, to be the rotamer most compatible with the four-stranded structure. These angles are standard staggered conformations and they are in agreement with the preferred rotamer angles of most leucines in the a and d positions in the less regular composition of the hydrophobic core of the Rop protein.

The refined model with the chosen leucine rotamers was
mutated to the sequence of 2H and 4H and these structures were again minimized with steps 1–3 of the procedure described above. The final unrestrained minimization was omitted, since the minimization with tethered backbone coordinates yielded structures of superior quality as observed in previous modeling calculations (Sönntigsen et al., 1995).

The procedure applied for the generation of models of the two-stranded antiparallel coiled-coils was identical to the one described above. The starting coordinates for the individual coils were obtained from the crystal structures of the coils GCN4 (pdb2zaa) (O’Shea et al., 1991) and seryl-tRNA synthetase (pdb1sry.ent) (Cusack et al., 1990) and the approximate relative alignment of the average coils was obtained by superposition with the backbone coordinates of seryl-tRNA synthetase (A35–A55 and A69–A91).

The models were analyzed at every stage using the in-house written software package VADAR, version 1.2 (Wishart et al., 1994) to assess the structural quality and identify packing defects. Surface accessible areas were calculated with the program ANAREA (Richmond, 1984) for each atom in the model using a probe radius of 1.4 Å and the set of van der Waals radii of Chothia (1976). These areas were summed by residue or character using the definition for hydrophobic/hydrophilic atoms by Chothia (1976) as implemented in VADAR (Wishart et al., 1994).

**Results**

**Peptide design**

The approach used in this study was to design two antiparallel, disulfide-bridged coiled-coil peptides that are identical to each other (Figure 1), except in the position of one Ala residue in the middle heptad which is indicated by an asterisk. In peptide 2H the two Ala residues in the middle heptad are in-register (the same rung) with respect to each α-helical chain, while peptide 4H contained two Ala residues in the middle heptad that are not in-register (different rungs). Thus, peptide 2H has a Leu-Ala-Leu-Ala hydrophobic packing in the middle heptad while peptide 4H has two Leu-Ala hydrophobic packings (Leu-Ala-Leu-Ala).

The CD spectra of the two disulfide-bridged peptides under benign conditions (Figure 2) show that the ratio of the negative molar ellipticities at 220 and 208 nm (220/208) was ~1.0 for both peptides, which has been suggested to be typical of interacting α-helices (Lau et al., 1984; Cooper and Woody, 1990). In the presence of 50% TFE the molar ellipticity ratio dropped to ~0.85 which, in turn, is characteristic of single-stranded α-helices. Taken together, these CD spectra indicate that both peptides were coiled-coil structures under benign conditions. In the presence of 50% TFE, which has been shown to disrupt tertiary and quaternary structure and promote secondary structure (Lau et al., 1984; Cooper and Woody, 1990; Sönntigsen et al., 1992), the α-helical chains in both peptides ‘open up’ into non-interacting α-helices. There was very little difference in the helical content determined at 220 nm for both peptides under benign conditions and in the presence of TFE (Figure 2A and B), which suggests that both peptides were already completely α-helical under benign conditions.

The apparent molecular weights of the peptides were determined by comparing the retention times of the peptides with coiled-coil standards of known molecular weights (Figure 3A). The retention time of peptide 2H was slightly shorter under benign conditions (Figure 3B) than in 6 M GdnHCl (Figure 3C).

![Fig. 3. (A) Plot of log mol. wt and retention time from the size-exclusion chromatography of coiled-coil standard peptides (O). The peptide standards were composed of disulfide-bridged homodimers with the following amino acid sequences: ac-EALK,EIEALK,EIEALK,EIEALK,EALK-am (38mer, 4041 Da), ac-KA,EIEALK,EIEALK,EIEALK,EALK-am (46mer, 5024 Da), ac-EALK,EIEALK,EIEALK,EIEALK,EALK-am (52mer, 5652 Da), ac-EALK,EIEALK,EIEALK,EIEALK,EALK-am (66mer, 7162 Da) and ac-EALK,EIEALK,EIEALK,EIEALK,EALK-am (70mer, 7676 Da). The last standard was in equilibrium with its dimer (140mer, 15 352 Da). SEC chromatogram of peptide 2H under (B) benign conditions and (C) in the presence of 6 M GdnHCl. SEC chromatogram of peptide 4H under (D) benign conditions and (E) in the presence of 3 M GdnHCl and (F) 6 M GdnHCl. The benzene buffer contained 50 mM phosphate, 0.1 M KCl, pH 7.0. The flow rate was 0.5 ml/min in all cases. The squares (•) in (A) represent the molecular weights of the four-stranded (15 400) and two-stranded (7800) coiled-coils.

| Table I. Comparison of the stability and molecular weights of peptides 2H and 4H |
|-----------------|-----------------|-----------------|-----------------|
| Peptide        | [Urea] (M)      | ΔG_H2O (kcal/mol) | Apparent molecular weights |
| 2H             | 4.1             | 2.9             | 7800 (9200) |
| 4H             | 5.3             | 5.4             | 15 400 (15 200) |

*Size-exclusion chromatography.

Sedimentation equilibrium experiments.

coiled-coil was slightly higher than the random coil form of the same peptide, shown previously (Hodges et al., 1981). This is probably due to its rod-like shape, where the axial (length-to-diameter) ratio of the two-stranded coiled-coil is larger than either that of a protein or random coil structure of the same molecular weight.

The more interesting observation was that the retention time of peptide 2H under benign conditions (Figure 3B) was ~4 min
A switch between two- and four-stranded coiled-coils

Fig. 4. (A) A plot of concentration versus radial distance from sedimentation equilibrium of peptide 2H performed at 26 000 r.p.m. The data were fit to a monomer ↔ dimer model, using the monomeric (two-stranded) molecular weight of 7422. The plot of residuals is shown in the upper panel and the best-fit curve shown in the bottom panel. (B) Similar plots from peptide 4H after fitting to a dimer (four-stranded) ↔ tetramer (eight-stranded) model.

longer than that of peptide 4H (Figure 3D). From comparison with the retention times of coiled-coil standards in size-exclusion chromatography (Figure 3A), the apparent molecular weights of peptides 2H and 4H were 7800 and 15 400 respectively (Table I). These results indicate that peptide 2H exists as a two-stranded coiled-coil while peptide 4H formed a four-stranded coiled-coil. The four-stranded structure of peptide 4H does not appear to be due to non-specific electrostatic interactions because the retention time in size-exclusion chromatography did not change in the presence of 1 M KCl (data not shown). In fact, the four-stranded form was so stable that in the presence of 3 M GdnHCl it was only partially denatured (Figure 3E). However, in 6 M GdnHCl peptide 4H was completely denatured and eluted at the same retention time (Figure 3F) as that of the denatured peptide 2H (Figure 3C). These results suggest that dissociation of the four-stranded coiled-coil to the two-stranded random coil is most likely a two-state transition under these conditions or at least the two-stranded coiled-coil intermediate is not present in any significant amount.

The results from sedimentation experiments were consistent with size-exclusion data. Peptides 2H and 4H gave apparent average molecular weights of 9200 and 15 200 respectively. After using non-linear analysis to fit the data to various association state models, the 2H peptide appeared to best fit a monomer ↔ dimer equilibrium model, giving an apparent $K_d$ of $3.3 \times 10^3$ M (Figure 4A). This result suggests that the monomeric (two-stranded) form of peptide 2H exists in equilibrium with its dimeric (four-stranded) state. On the other hand, peptide 4H did not fit the monomer ↔ dimer equilibrium model using the two-stranded form as the monomeric unit. However, using the dimer (four-stranded) ↔ tetramer (eight-stranded) model, a good fit was obtained, resulting in an
The fraction of peptide in the folded state was calculated from the four-stranded form is a very stable structure. The results show that the two-stranded form of peptide 4H does not exist in a significant amount relative to the four-stranded form, that is the four-stranded form is a very stable structure. The results also suggest that the four-stranded form of peptide 4H exists in equilibrium with its eight-stranded species, the structure of which is unknown, but is suspected to be a loose association of two four-stranded forms without rearrangement of the hydrophobic interface. However, under the typical peptide concentration of ~100 µM used in the experiments, calculations indicate that peptide 2H exists as 68% two-stranded, while peptide 4H exists as 83% four-stranded.

Urea was chosen as the chemical denaturant because its values reflect the overall stability of the folded structure, that is the sum of the contributions from the hydrophobic and electrostatic interactions (Monera et al., 1994b). The urea denaturation curve for both peptides 2H and 4H showed smooth denaturation profiles with one transition point (Figure 5). The [urea]_1/2 values were 4.1 versus 5.3 M for the two-stranded and four-stranded coiled-coils respectively (Table I). However, the [denaturant]_1/2 values have to be interpreted with caution because the bimolecular unfolding of peptide 4H is concentration dependent (Bowie and Sauer, 1989; De Francesco et al., 1991; Neet and Timm, 1994) while the monomolecular unfolding of peptide 2H is not. A more accurate alternative is to compare their free energy of unfolding in the absence of denaturant, ΔG°_H2O, from the linear extrapolation method (Schellman, 1978; Pace, 1986), where the concentration effect in peptide 4H was included in the calculations. In addition to the linear extrapolation method, which in practice usually includes only the data points around the transition region, we used a non-linear curve-fitting equation to calculate the ΔG°_H2O based on all data points. Figure 5 and Table I show that the ΔG°_H2O is higher for the four-stranded coiled-coil (peptide 4H, 5.4 kcal/mol) than for the two-stranded coiled-coil (peptide 2H, 2.9 kcal/mol). The linear extrapolation method gave very similar results.

The temperature denaturation results (Figure 6) also show that peptide 4H is more stable than 2H, consistent with the results from urea denaturation studies. However, the actual t_m values have to be interpreted with caution because of the concentration dependence of the t_m for peptide 4H. An important result from the CD wavelength scans at different temperatures is the presence of a well-defined isodichroic point at ~203 nm, which has been suggested to be indicative of a two-state transition (Engel et al., 1991; Thompson et al., 1993). Thus, these results support the validity of the assumptions made for the two-state denaturation for both peptides 2H and 4H.

Discussion
In spite of the increasing indication that hydrophobic interactions play a major role in controlling the degree of association in coiled-coils (Alber, 1992; Harbury et al., 1993, 1994; Lovejoy et al., 1993; Zhu et al., 1993), it is still not clear how these hydrophobic interactions favor one state of aggregation over another. In this study, we are showing that the equilibrium can be shifted towards the two-stranded coiled-coil by destabilizing the four-stranded coiled-coil assembly.

The question arises as to why four-stranded coiled-coils are formed in the first place. A possible driving force towards the formation of a four-stranded coiled-coil is the optimization of the burial of the hydrophobic residues in the four-stranded coiled-coil, which effectively shields the hydrophobic core from hydration. Results from molecular modeling studies showed that optimum packing of the four strands is achieved by the tilting of the individual chains by ~45°, as was
Table II. Estimated buried surface areas (Å²) from molecular modeling studies

<table>
<thead>
<tr>
<th>Surface type</th>
<th>Total surface area</th>
<th>Buried surface areaa</th>
<th>Peptide 2H</th>
<th>Peptide 4H</th>
<th>Peptide 4H</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Two-stranded</td>
<td>Four-stranded</td>
<td>Two-stranded</td>
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<td>4368 (69)</td>
<td>3153 (50)</td>
<td>4296 (69)</td>
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<td>2509 (63)</td>
<td>2083 (52)</td>
<td>2505 (63)</td>
</tr>
<tr>
<td>Total</td>
<td>10 331</td>
<td>4970 (48)</td>
<td>6877 (67)</td>
<td>5236 (51)</td>
<td>6901 (67)</td>
</tr>
</tbody>
</table>

The numbers in parentheses are the percentages of buried surface area relative to the total surface area.

a Represents the total surface area of the unfolded two-stranded monomer.

b Calculated by subtracting the accessible surface area of each folded structure from the total surface area, where all values are normalized on the basis of the unfolded two-stranded monomer.

Fig. 7. (A) A cross-section schematic representation of the middle heptad of the two-stranded antiparallel coiled-coil (peptide 2H). Looking from the top, the N-terminal end of one chain propagates downward while the C-terminal end of the other chain propagates downward. The arrow in the backbone indicates the N→C direction of chain propagation. Thus, N2 and C2 represent the N- and C-terminal ends of peptide 2H respectively. The heavy dashed line represents the disulfide bridge between Cys2 and Cys33. The solid and dashed double arrows represent interchain electrostatic attractions and intrachain electrostatic repulsions respectively. Hydrophobic packings are indicated between Leu→Leu (dashed double arrow) and the Ala→Ala (open double arrow). Where applicable, the same designations are used in (B), (D) and (E). (B) The cross-section schematic representation of the potential four-stranded coiled-coil. In this structure all adjacent α-helical chains are antiparallel to each other and both disulfide-bridged ends are on the same side (top) of the four-stranded coiled-coil. C2'→N2' are used to indicate that this two-stranded unit is identical to C2→N2. Only one central hydrophobic plane is shown for simplicity, as shown by the open double arrows. (C) Relative arrangement of the Ala and Leu residues in the middle heptad of the four-stranded coiled-coil. A and L represent the Ala and Leu residues respectively. (D), (E) and (F) represent the corresponding structures for peptide 4H and the same symbols are used as in (A), (B) and (C).
also previously observed from X-ray crystallographic studies (Harbury et al., 1993). As a consequence of this tilting of individual strands the estimated buried surface area increased for both peptides 2H (from 48 to 67%) and 4H (from 51 to 67%) when each was modeled as two-stranded and four-stranded coiled-coils respectively (Table II). These represent an increase of ~35% in buried surface area, which is slightly higher than the 25% previously calculated from X-ray crystallographic data of other coiled-coils (Harbury et al., 1993). The discrepancy might be model-specific and could originate from the modeling procedure in vacuo (Chiche et al., 1989).

However, it is obvious that the increase in buried surface area in going from a two-stranded to a four-stranded coiled-coil cannot be used to explain why peptide 2H is two-stranded while peptide 4H is four-stranded. The cross-sectional diagrams of these coiled-coils are shown in Figure 7(A) and (E) respectively. The surface area estimates are almost identical for both peptides, either in the two-stranded or four-stranded forms (Table II). The difference in structure, as well as in stability, between peptides 2H and 4H must, therefore, be due to the difference in the size of internal cavities formed in the potential four-stranded structure. Molecular modeling of peptide 2H as it exists in the two-stranded form showed that the residues are well packed, providing a large contact surface area in the hydrophobic core which is continuous along the interface, except with the two Ala residues in the middle heptad which decreased the hydrophobic contact area in this region (Figure 8A). If this peptide were to form the four-stranded coiled-coil, the four Ala residues in the same rung of the middle heptad of each α-helical chain would lie in the
packing, both in the two-stranded (Figure 8B) and four-stranded coiled-coils, but the latter is preferred because of greater buried surface area. The ability of the Rop protein relaxed substantially and atoms moved into part of the cavity (Figure 8D). Thus, the hypothetical four-stranded structure of peptide 2H is not favorable, but it would be even more energetically unfavorable for the individual chains to exist either in random coil conformation or as extended α-helices, where in each case their hydrophobic residues are fully exposed to the solvent. Therefore, it appears that the two-stranded coiled-coil, where the two chains are disulfide bridged, is the most stable structure that predominates under benign conditions.

In contrast, peptide 4H does not predominate in the two-stranded form and favors the four-stranded coiled-coil, the cross-sections of which are shown in Figure 7(D) and (E) respectively. This is presumably because in the four-stranded form the four Ala residues are in two different planes, which results in alternating Leu-Ala hydrophobic packing in the middle heptads (Figure 7F). The potential cavity is distributed over a wider region and may even be partially filled by relatively minor adjustments of the neighboring Leu side chains to compensate for the small side chains of Ala residues. This interpretation is consistent with previous observations that in some T4 lysozyme mutants with Leu → Ala replacements the protein relaxed substantially and atoms moved into part of the space vacated by the Leu side chain (Eriksson et al., 1992). Indeed, space filling models indicate that this alternating Leu-Ala hydrophobic packing in the two planes provides adequate packing, both in the two-stranded (Figure 8B) and four-stranded (Figure 8C) coiled-coils, but the latter is preferred because of greater buried surface area. The ability of the Leu-Ala pairs to maintain a stable four-helix structure was previously demonstrated when an analog of the Rop protein where the hydrophobic interface exclusively contained Leu-Ala pairs was shown to be active (Munson et al., 1994).

The four-stranded structures are based on the assumption that each strand is antiparallel to its nearest neighbor. This antiparallel orientation is consistent with theoretical calculations of the backbone electrostatic interactions between helices in a four-stranded structure (Chou et al., 1984, 1988; Hol, 1985; Rey and Skolnick, 1993), as well as native protein conformations (Weber and Salemme, 1980; Richardson, 1981; Banner et al., 1987; Cohen and Parry, 1990). In these peptide models, two all-antiparallel orientations are possible: either the disulfide bridges are on the same end or on opposite ends of the four-stranded coiled-coil. However, the former is presumed to be the preferred structure because the latter would result in 10 pairs of interchain electrostatic repulsions from glutamic acid residues, which had been previously shown to be unfavorable for coiled-coil formation (Monera et al., 1993, 1994a; Zhou et al., 1994; Kohn et al., 1995a,b).

The results from urea and temperature denaturation studies clearly show that, calculated on a monomer (two-stranded) basis, the four-stranded peptide 4H is 2.5 kcal/mol more stable than the two-stranded peptide 2H (Figures 5 and 6 and Table I). This difference in the structure and stability of peptides 2H and 4H cannot be explained in terms of the difference in residue hydrophobicity in the coiled-coil interface, since they have identical amino acid sequence except in single Ala positions in the middle heptads. Again, we rationalize these results in terms of how the peptide responds to the potential cavity formed from Leu → Ala substitutions. When peptide 2H was modeled as a four-stranded structure (Figure 9) the resulting cavity was estimated to have a surface area of 177 Å² and volume of 187 Å³, large enough to accommodate five to 10 water molecules. Similarly, based on the estimated destabilization energies of 20 kcal/mol Å² and 24 kcal/mol Å³ (Matthews, 1993), formation of this cavity corresponds to a total destabilization of 3.5–4.5 kcal/mol for the dimeric structure (four-stranded) or 2 kcal/mol per monomeric coiled-coil (two-stranded). The 2.5 kcal/mol difference in stability between peptides 2H and 4H probably reflects the sum of the effects...
due to the difference in the sizes of the cavities and the different amounts of buried surface areas as a consequence of their two-stranded and four-stranded existence. It had been estimated that each Leu → Ala substitution would correspond to a minimum of 1.9 kcal/mol in destabilization energy (Matthews, 1993) or a total destabilization of 7.6 kcal/mol for the Leu → Ala substitutions in the four chains of a four-stranded coiled-coil. We wanted to verify this by synthesizing a similar peptide analog that had Leu in all α and ß positions as a reference peptide. As expected, this control peptide was found to be highly helical and four-stranded. However, it was too stable for our purpose, retaining ~80% helical content in the presence of 8 M urea and 95% helical content at 85°C under benign buffer conditions. Nevertheless, these results confirm the superiority of an all-Leu packing compared to either type of Leu-Ala substitutions. We can also postulate that the ΔΔG_H2O between the control and the 4Leu → 4Ala substitutions would be lesser when the four substituted alanine residues are localized in the different rungs than when they are all in the same rung of the potential four-stranded coiled-coil structure. This supports our contention that the favored two-stranded coiled-coil structure of peptide 2H is a consequence of the destabilizing effect of the cavity formed in its potential four-stranded structure when the four Ala are localized in the same plane.

These results have shed some light on the general notion that hydrophobic interactions are the more important driving force in protein folding (Dill, 1990; Matthews, 1993). However, the strength of the hydrophobic effect alone, that is the energy of stabilization provided by the transfer of hydrophobic surfaces from a solvent to the interior of a protein (Chothia, 1976; Richards, 1977), is not sufficient to explain the differences in folding and stability between peptides 2H and 4H. These two peptides have identical amino acid composition and secondary structure. The results are more consistent with the assertion that it is the rigid parts of the proteins, such as the hydrophobic core, that are critical for folding and stability (Matthews, 1993). A very subtle positional effect of a single alanine residue can have a very dramatic effect on the state of molecular aggregation. In more specific terms, substitution of Ala for a large hydrophobe in the interface may not be enough to destabilize a four-stranded coiled-coil, but proper positioning of the substituted Ala residues has dramatic effects on the state of association. This technique is a simple but important design element that can be used to switch an undesired four-stranded coiled-coil assembly (consisting of two disulfide-bridged two-stranded monomers) into a two-stranded coiled-coil.

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References

A switch between two- and four-stranded coiled-coils


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