Electrostatic Interactions Control the Parallel and Antiparallel Orientation of α-Helical Chains in Two-Stranded α-Helical Coiled-Coils†

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ABSTRACT: The role of interchain electrostatic interactions in orientating α-helical chains to form two-stranded parallel and antiparallel coiled-coils has been investigated. Four disulfide-bridged coiled-coils were designed: parallel coiled-coils with interchain electrostatic attractions (P/A) and repulsions (P/R) and antiparallel coiled-coils with interchain electrostatic attractions (AP/A) and repulsions (AP/R). These coiled-coils were made by air oxidation of two 35-residue peptides with the appropriate heptad repeat (LaEbAcLdEeGfKg or LaAbEcLdKeGfEg) to give the desired interchain electrostatic interactions, and the appropriate position of the cysteine residue (C2 or C33) to give the desired chain orientation. The coiled-coils were characterized by circular dichroism spectroscopy, and their stabilities were assessed by guanidine hydrochloride and urea denaturations. The results indicated that the favored chain orientation, that is, the major disulfide-bridged product formed under benign conditions, was the one that provides interchain electrostatic attractions between oppositely-charged amino acid residues in the e–g′ and g–e′ positions of the parallel coiled-coil and the g–g′ and e–e′ positions in the antiparallel coiled-coil. When the electrostatic interactions were similar, the antiparallel coiled-coils were more stable than the parallel coiled-coils. However, the overall stability of the coiled-coils was either increased by interchain electrostatic attractions or decreased by interchain electrostatic repulsions, as determined by urea denaturation. Thus, the order of overall stability of these coiled-coils was AP/A > P/A > AP/R > P/R. This study demonstrates the importance of interchain electrostatic interactions in determining the parallel or antiparallel orientation of α-helical chains in two-stranded coiled-coils.

The two-stranded coiled-coil motif is characterized by two amphipathic α-helical chains wrapping around each other into a left-handed superhelix (Crick, 1953). Although there are two possible orientations of the α-helical chains to form a coiled-coil, parallel or antiparallel, a particular coiled-coil exists only in one specific orientation. Coiled-coils in fibrous proteins such as tropomyosin (Johnson & Smillie, 1975; Lehrer, 1975; Stewart, 1975; McLachlan & Stewart, 1975) and the dimerization domain of DNA binding proteins (O'Shea et al., 1989a,1991) have parallel orientations. However, when the α-helical chain turns back onto itself to form a coiled-coil, such as in the long coiled-coil extension of the *Escherichia coli* seryl-tRNA synthetase (Cusack et al., 1990), chain orientation becomes antiparallel. In addition, α-helical chains that form multiple helix bundles or similar associations in native proteins are known to have both parallel and antiparallel orientations (Weber & Salemme, 1980; Richardson, 1981; Banner et al., 1987; Cohen & Parry, 1986, 1990; Parry et al., 1992; Lovejoy, 1993).

While the specificity of the knobs-into-holes packing of the hydrophobic residues in the coiled-coil interface favors an orientation where the two α-helices pack approximately 20° away from parallel, the two α-helices could also potentially run in opposite directions (antiparallel) and maintain a similar knobs-into-holes packing of the hydrophobes (Crick, 1953). We have recently shown that when a pair of peptides that normally form a parallel coiled-coil were modeled as an antiparallel coiled-coil, there were no restrictions to the packing of the hydrophobes, and the stability data showed that the antiparallel orientation appeared to be preferred (Monera et al., 1993). However, the observation that the antiparallel coiled-coil did not form spontaneously in significant amount in benign medium suggests that other interactions must be important in determining the orientation of the polypeptide chains and overriding the preferred hydrophobic packing of the antiparallel orientation.

What are the interactions that control the parallel or antiparallel orientation of the α-helical chains in two-stranded coiled-coils? In addition to the periodicity of hydrophobes in the a and d positions of the tropomyosin sequence (Hodges et al., 1972; Sodek et al., 1972), an apparent periodicity in the occurrence of charged amino acid residues in the e and g positions had also been observed (Stone et al., 1975; McLachlan & Stewart, 1975). These authors suggested that these amino acids were involved in interchain electrostatic interactions that were important in the alignment of the α-helical chains (in-register versus staggered), as well as for the stability of the coiled-coil structure. This view is consistent with the observation that, in general, positions e and g of naturally occurring two-stranded parallel coiled-coils are usually occupied by charged amino acid residues that could provide potential electrostatic attractions between opposite α-helical chains which, in turn, were presumed to stabilize the coiled-coils (Parry, 1975, 1981; Parry & Fraser, 1985; Cohen & Parry, 1986; Conway & Parry, 1988, 1990; O'Shea et al., 1989b,1991, 1992; Schuermann et al., 1991; Ellenberger et al., 1992; Hu & Sauer, 1992; Adamson et al., 1993). Similarly, in antiparallel coiled-coils (Cusack et al., 1990), in the three-helix motif (Banner et al., 1987; Parry et al., 1992; Lovejoy

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et al., 1993), or in four-helix bundles (Weber & Salemme, 1980; Cohen & Parry, 1990; Paliakasis & Kokkinidis, 1992) the charged residues in the e and g positions of the interacting helices are also generally favorable for interchain ionic attractions. As a result, interchain electrostatic attractions have become integrated in most designs and modeling of coiled-coils (Hodges et al., 1981, 1988, 1990; Lau et al., 1984; DeGrado et al., 1989; Cohen & Parry, 1990; Semchuk et al., 1990; Engel et al., 1991; Graddis & Chaiken, 1992; Graddis et al., 1993; Zhou et al., 1992a-d, 1993; Zhu et al., 1992, 1993; Lovejoy et al., 1993). While there seems to be a general acceptance of the role of electrostatic interactions in stabilizing coiled-coil structures, there is essentially no experimental data concerning the role of interchain electrostatic interactions in controlling the parallel or antiparallel orientation of the α-helical chains to form a coiled-coil.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification. The starting peptides were synthesized using solid-phase methodology and purified by reversed-phase HPLC,[1] and their identities were confirmed by amino acid analysis and by mass spectrometry as described previously (Monera et al., 1993).

Air Oxidation and Purification. To test for spontaneity of formation of heterostranded coiled-coils under benign conditions, small-scale oxidation of the reduced peptides was carried out by dissolving a mixture of 0.5 mg of each peptide in 1.0 mL of 100 mM NH_4HCO_3, pH 8.3, and stirring the solution magnetically in an open vial at room temperature (25 °C). The progress of oxidation was monitored by injecting 50-μL aliquots of the reaction mixture into a Hewlett-Packard HP-1090 liquid chromatograph equipped with an analytical C8 column (Zorbax 300SB-C8, 4.6 mm × 25 cm, 5-Å particle size, 300-Å pore size, Du Pont). The oxidation products were eluted at 1 mL/min using a linear AB solvent gradient of 0.5% 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.

For large scale-oxidation, 7.5-mg samples of each reduced peptide were dissolved together and magnetically stirred in 3 mL of 100 mM NH_4HCO_3, pH 8.3. For peptide combinations that did not spontaneously form heterostranded products, the oxidation was carried out in the same buffer containing 6 M GdnHCl. To separate and purify the oxidized products, the peptide solutions were neutralized with dilute acetic acid and then injected into a Varian Series 5000 liquid chromatograph equipped with a semipreparative reversed-phase C_{18} column (Synchropak RP-P, 10 mm × 25 cm i.d., 6.5-μm particle size, 300-Å pore size, SynChrom, Lafayette, IN). 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% trifluoroacetic acid in water and solvent B was 0.05% trifluoroacetic acid in acetonitrile. Each of the disulfide-bridged homostranded products in the HPLC chromatogram was identified by spiking the samples with independently prepared disulfide-bridged peptides, and the third products were designated as the heterostranded disulfide-bridged peptides. In all cases, the identities of the products were confirmed by amino acid analysis, mass spectrometry, and reduction and HPLC characterization of the two reduced peptides.

[1] Abbreviations: CD, circular dichroism; DTT, dithiothreitol; GdnHCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; TFE, 1,1,1-trifluoroethanol.

To investigate the effect of salts on the peptide combinations that did not spontaneously form heterostranded products under benign conditions, small-scale oxidations were also carried out in 50 mM TRIS buffer at pH 8.3 in the presence of 0.5 M $NaClO_4$ and 1 M $MgCl_2$.

Circular Dichroism Spectroscopy. Stock peptide solutions (~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 at pH 7). Two sets of peptide solutions were prepared for CD studies of each peptide: one was in benign buffer (10 μL of stock peptide solution was diluted with 50 μL of the same buffer), and the other was in 50% TFE (10 μL of stock peptide was diluted with 20 μL of buffer and 30 μL of TFE). The peptide solutions were then loaded into a 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, ver. 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-camphorsulfonate at 290.5 nm. Ellipticity is reported as mean residue ellipticity (θ), and the limit of error of measurements at 220 nm was ±300 deg cm^2 dmol^{-1}.

Denaturation Studies. Stock GdnHCl (8 M) and urea (10 M) solutions were prepared in 50 mM phosphate at pH 7. Then, aliquots of these denaturants were used to prepare a series of solutions containing different concentrations of GdnHCl and urea. For urea denaturations in the presence of salts, the urea stock solution (11 M) was prepared in 50 mM TRIS containing 0.5 M $NaClO_4$ and 1 M $MgCl_2$ at pH 8.3.

Size-Exclusion Chromatography. About 0.5 mg of disulfide-bridged peptides was dissolved in 0.5 mL of 50 mM TRIS, pH 7, containing 0.5 M $NaClO_4$ and 1 M $MgCl_2$. A 50-μL aliquot was injected into a Hewlett-Packard Model HP-1090 liquid chromatograph equipped with a Beckman 2000SW size-exclusion column and eluted with 50 mM TRIS/0.5 M $NaClO_4$/1 M $MgCl_2$, pH 7, at 0.5 mL/min.

RESULTS AND DISCUSSION

The difficulty in interpreting protein folding and stability data lies in our limited understanding of both complex protein structures (Dill, 1990) and the solvent denaturation process (Pace, 1986; Dill, 1990; Makhatadze & Privalov, 1992; Creighton, 1993). In this report, we addressed the first problem by using synthetic model proteins with only one type of secondary structure, the two-stranded α-helical coiled-coil; the latter, by using two types of denaturants, GdnHCl and urea, which differ in their modes of denaturation.

This study was based on the premise that the relative amount of disulfide-bridged product formed under benign conditions (100 mM NH_4HCO_3, pH 8.3) is proportional to the propensity of the two α-helices to orient either in parallel or antiparallel fashion in forming the coiled-coil. In the design of the four disulfide-bridged coiled-coils, the spatial orientations of the hydrophobic residues were kept similar so that they differ mainly in chain orientation and in interchain electrostatic interactions. These coiled-coils were formed by air oxidation of an appropriate combination of any two of the starting reduced peptides, each containing a 5-heptad repeat with the amino acid sequence LEALEGK or LAELKGE. To facilitate

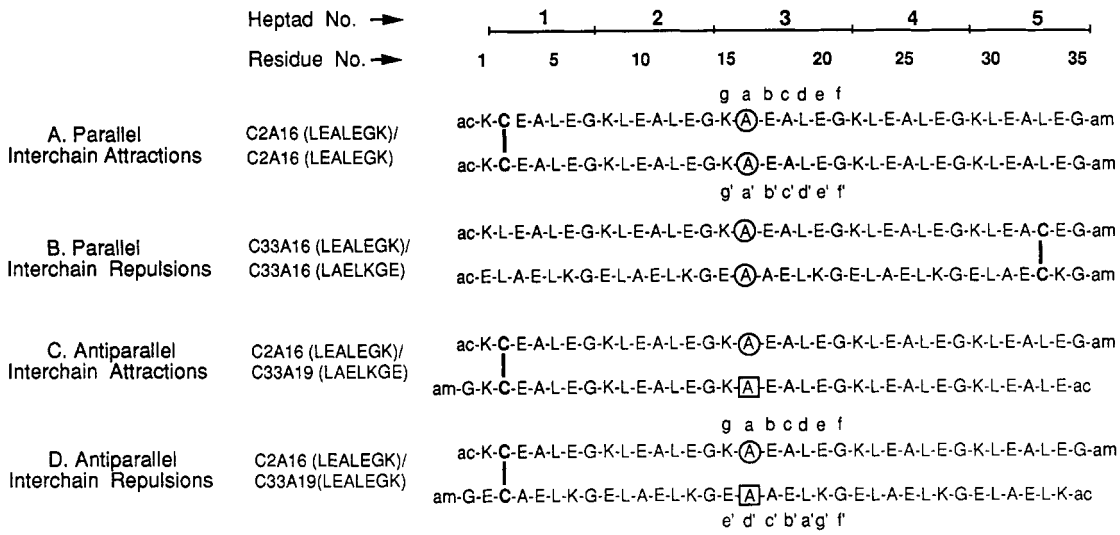

FIGURE 1: The amino acid sequences of the five peptides used for forming the four disulfide-bridged coiled-coils. Different combinations of the reduced peptides were disulfide-bridged to form P/A, P/R, AP/A, and AP/R. The names of the disulfide-bridged peptides contain the names of the two peptides that were used to form the coiled-coil. In the parallel coiled-coils the amino acid sequences of the two α-helical chains run in the same direction and are in register, as shown in (A). In the antiparallel coiled-coils, the two α-helical chains run in opposite directions, which results in changes in the pairing of amino acid residues in the coiled-coil, as shown in (D). The Ala residues are circled at position 16 and boxed at position 19.

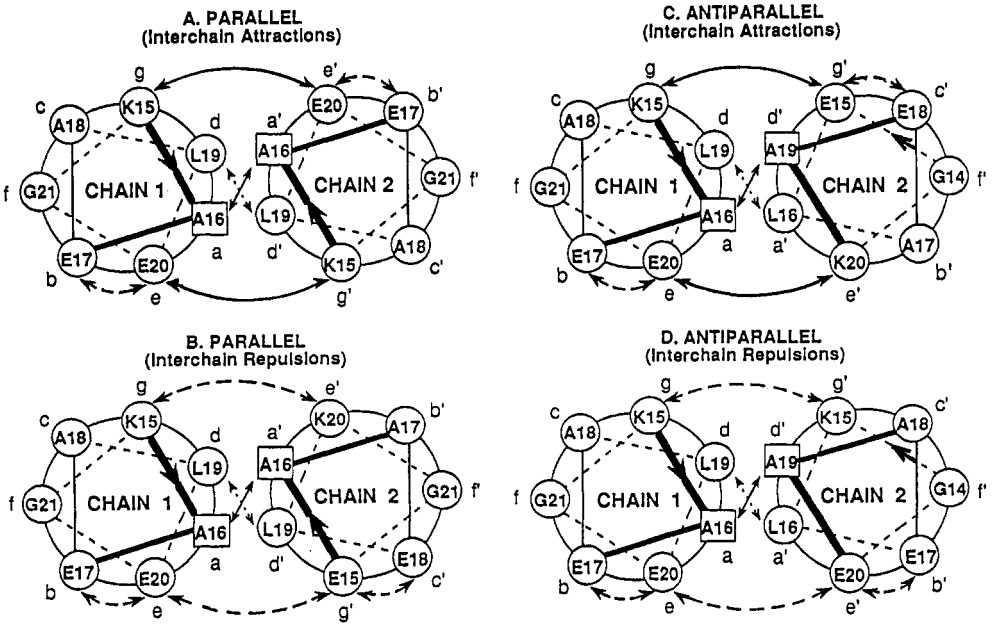

FIGURE 2: Cross sections of the third (middle) heptads of the disulfide-bridged coiled-coils. Potential electrostatic repulsions are indicated as dashed arrows, and electrostatic attractions are indicated as solid arrows. In the parallel coiled-coils, the direction of chain propagation (N → C) are the same for both chains and goes away from the reader, into the paper. In the antiparallel coiled-coil, the directions of chain propagation are opposite; that is, chain 1 goes into the paper, while chain 2 comes out of the paper, toward the reader. The amino acid residues in chain 2 are indicated as primes (′). The A16a–A16a′ and A16a–A19d′ pairs are shown in boxes to indicate that they are on top (toward the reader) of the L19d–L19d′ and L19d–L16a′ pairs, respectively.

disulfide bridge formation, Leu at either position 2 or 33 was substituted with Cys (C2 or C33, respectively). When the disulfide bonds are located at the ends of the coiled-coil (positions 2a or 33d), the effects on stability are similar (Zhou et al., 1993). A single Leu → Ala substitution at either position 16 or 19 was necessary in order to bring the stabilities of the disulfide-bridged peptides into a range that is convenient for GdnHCl and urea denaturations. Therefore, to reflect both the positions of the cysteine and alanine residues and the sequence of the heptad repeat, the five starting peptides are designated as C2A16(LEALEGK), C33A16(LEALEGK), C33A19(LEALEGK), C33A16(LAELKGE), and C33A19-(LAELKGE). Consequently, the four disulfide-bridged coiled-coils are as follows: parallel with interchain electrostatic attractions [P/A, C2A16(LEALEGK)/C2A16(LEALEGK)] and repulsions [P/R, C33A16(LEALEGK)/C33A16-(LAELKGE)] and antiparallel with interchain electrostatic attractions [AP/A, C2A16(LEALEGK)/C33A19 (LAELKGE)] and repulsions [AP/R, C2A16(LEALEGK)/ C33A19(LEALEGK)] (Figure 1). Figure 2 shows representations of these coiled-coils in cross section.

When an appropriate pair of reduced starting peptides was subjected to complete air oxidation, only three disulfide-bridged products were expected (Figure 3B–F), one homostranded (Hom) coiled-coil from each starting peptide and a heterostranded (Het) coiled-coil that resulted from the combination of the two different starting peptides. In Figure 3A, only one product was possible, a homostranded parallel

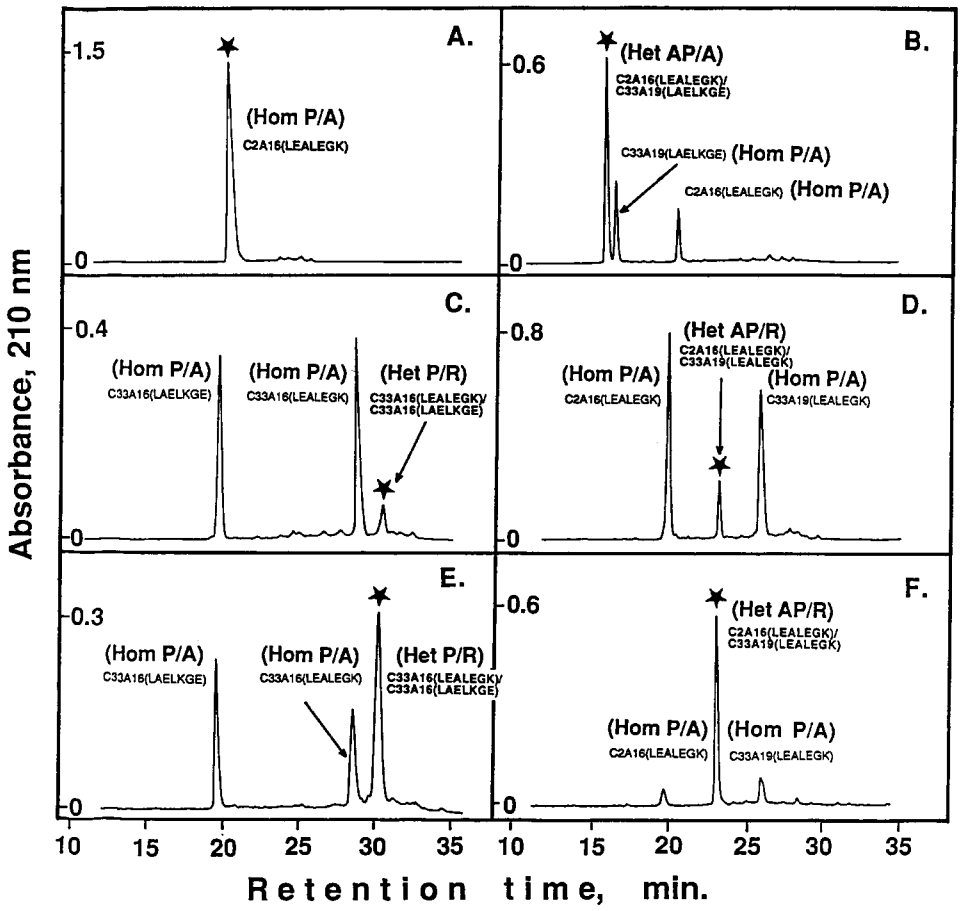

FIGURE 3: HPLC chromatograms of the products of the oxidation of a mixture of peptides to form the coiled-coils with the desired alignment and interchain electrostatic interactions. All peaks correspond to disulfide-bridged (oxidized) products. For example, C2A16(LEALEGK) corresponds to a disulfide-bridged homostranded parallel coiled-coil (HomP/A), and C33A16(LEALEGK)/C33A16(LAELKGE) corresponds to a disulfide-bridged heterostranded parallel coiled-coil (Het P/R) of C33A16(LEALEGK) and C33A16(LAELKGE) peptides. The peaks of interest are indicated by a star (★). In panels A, B, C, and D the air oxidation was carried out in 100 mM NH_4HCO_3, pH 8.3. In panels E and F the air oxidation was performed in 50 mM TRIS buffer, pH 8.3, in the presence of 0.5 M $NaClO_4$ and 1 M $MgCl_2$ (see Experimental Procedures).

coiled-coil. By design, all disulfide-bridged homostranded peptides have parallel chain orientation and have interchain electrostatic attractions, but the disulfide-bridged heterostranded peptides (Figure 2) may have either interchain electrostatic attractions or repulsions.

The circular dichroism spectra of the four peptides were very similar to each other, both under benign conditions (Figure 4A) and in the presence of 50% TFE (Figure 4B). Their very high molar ellipticities at 220 nm ($[\Theta]_{220}$) under benign conditions (50 mM phosphate, 0.1 M KCl, pH 7), which did not increase in the presence of a helix-inducing solvent such as TFE, suggest that all four disulfide-bridged peptides exist in a totally α-helical structure. Under benign conditions the $[\Theta]_{220}/[\Theta]_{208}$ ratio is very close to 1.00 (Table 1), which was previously proposed as diagnostic of a coiled-coil structure (Lau et al., 1984). In the presence of 50% TFE, which is known to disrupt tertiary and quaternary structures, the $[\Theta]_{220}/[\Theta]_{208}$ ratio decreased to around 0.9 (Table 1), which was also previously associated with extended or non-interacting α-helices (Lau et al., 1984; Cooper & Woody, 1990). These observations suggest that all four peptides exist in a coiled-coil structure under benign conditions. Size-exclusion chromatography in 50 mM TRIS, 1.0 M $MgCl_2$, and 0.5 M $NaClO_4$, pH 7, showed that all four coiled-coils had similar retention times, corresponding to the two-stranded 70-residue disulfide-bridged coiled-coils (data not presented).

Parallel versus Antiparallel Coiled-Coils. The relative distribution of disulfide-bridged products formed under benign

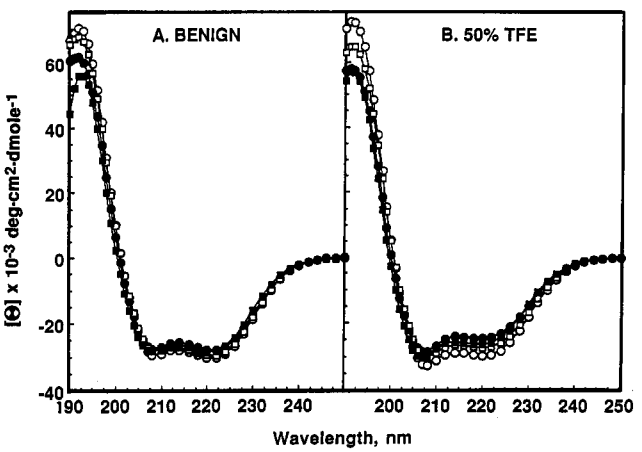

FIGURE 4: Circular dichroism spectra of the coiled-coils under benign conditions (A) and in the presence of 50% TFE (B): ○, P/A; ●, P/R; □, AP/A; ■, AP/R.

conditions suggests that the type of interchain electrostatic interactions determines whether the two α-helical chains orientate in a parallel or antiparallel fashion. For example, the formation of the homostranded parallel coiled-coil with interchain electrostatic attractions (HomP/A, Figure 3A) was more than 90% complete within the first hour of oxidation, suggesting that this product was readily formed. In addition, the antiparallel coiled-coil designed to have interchain electrostatic attractions (Het AP/A, Figure 3B) was also the

Table 1: Ellipticities and Stabilities of the Four Disulfide-Bridged Coiled-Coil Peptide Analogs

nomenclature (symbol)	coiled-coil alignment	interchain electrostatic interactions	$([\Theta]_{220})^a$ (deg cm^2 dmol^{-1})		$[\Theta]_{220}/[\Theta]_{208}$ Ratio		[denaturant]$_{1/2}{}^b$			$t_R{}^e$ (min)
			benign	50% TFE	benign	50% TFE	GdnHCl (M)	urea^c (M)	urea^d (M)	
P/A (○)	parallel	attractions	−30 450	−29 060	1.01	0.93	2.6	3.1	7.0	19
P/R (●)	parallel	repulsions	−28 090	−24 700	1.01	0.86	2.6	0.8	7.3	30
AP/A (□)	antiparallel	attractions	−29 680	−27 600	1.05	0.91	4.0	4.2	9.5	15
AP/R (■)	antiparallel	repulsions	−30 100	−27 800	1.01	0.88	3.6	1.4	8.2	23

[a] $[\Theta]_{220}$ is the calculated molar ellipticity of the coiled-coils at 220 nm. [b] The $[GdnHCl]_{1/2}$ and $[urea]_{1/2}$ values represent the concentration of denaturant at which 50% of the peptide is unfolded. [c] $[urea]_{1/2}$ values determined in the presence of a benign buffer (50 mM PO_4, 0.1 M KCl, pH 7.0). [d] $[urea]_{1/2}$ values determined in the presence of a benign buffer (50 mM PO_4, 0.1 M KCl, pH 7.0) containing 0.5 M $NaClO_4$ and 1.0 M $MgCl_2$. [e] t_R is the retention time of the coiled-coil on an analytical Zorbax 300SB-C8 column using a linear AB gradient of 25% B to 45% B at 0.5% B/min, where buffer A is 0.05% TFA in acetonitrile and buffer B is 0.05% TFA in water.

predominant product. In contrast, both the parallel (HetP/R, Figure 3C) and the antiparallel (HetAP/R, Figure 3D) coiled-coils with interchain electrostatic repulsions were formed only as minor products under benign conditions.

These data may be interpreted in terms of interchain electrostatic repulsions between similarly charged amino acid residues in the e–g′ and g–e′ positions of the parallel coiled-coil and the g–g′ and e–e′ positions in the antiparallel coiled-coil (Figure 2), hindering the two chains from coming together close enough to facilitate hydrophobic interactions. Thus, the coiled-coils with 10 pairs of interchain electrostatic attractions (5 Lys–Glu pairs on each side of the interface) were preferentially formed (P/A and AP/A), while those with 10 pairs of interchain electrostatic repulsions (5 Lys–Lys on one side and 5 Glu–Glu on the other) were not (P/R and AP/R). However, once a disulfide bridge is formed between a pair of peptides with interchain electrostatic repulsions, a stable coiled-coil can be formed.

It is interesting to note that in Figure 3B, where all the products have interchain electrostatic attractions, the ratio of HetAP/A to both HomP/A products (4:1:1) was higher than the 2:1:1 expected from purely random combination. In addition, the ratio of HetAP/R relative to both Hom P/A products increased from 0.2:1:1 under benign conditions (Figure 3D) to 9:1:1 in the presence of salt (Figure 3F). These observations indicate that when interchain electrostatic interactions are all attractions or when the attractions and repulsions are masked, the antiparallel product is favored over the parallel products, as previously suggested (Monera et al., 1993). Similarly, when all the potential products are parallel, the ratio of Het to both Hom/PA products only increased from 0.2:1:1 under benign conditions (Figure 3C) to the 2:1:1 ratio for random combination (Figure 3E) in the presence of salt to mask the electrostatics.

The GdnHCl denaturation data suggests that the more stable hydrophobic packing observed in the disulfide-bridged antiparallel coiled-coils (Table 1) was not the dominating factor in controlling the orientation of the polypeptide chains during the oxidation reaction. For example, while AP/R had a higher $[GdnHCl]_{1/2}$ (3.6 M) than P/A (2.6 M), P/A was spontaneously formed under benign conditions, while AP/R was not. However, the combined effects of hydrophobic interactions and the stabilizing or destabilizing effects of interchain electrostatic interactions, as measured in urea for these disulfide-bridged coiled-coils, does correlate with the preferred chain orientation. The products with higher overall stability, AP/A and P/A, with $[urea]_{1/2}$ values of 4.2 and 3.1 M, respectively (Table 1), were spontaneously formed, while the less stable AP/R and P/R, with $[urea]_{1/2}$ values of 1.4 and 0.8 M, respectively (Table 1), were not. Taken together, these results suggest that while the contribution of electrostatic interactions to protein stability is considered to be generally less than those from hydrophobic interactions (Dill, 1990; Hu et al., 1993), electrostatics appear to be the dominant determinants of specificity of dimerization in terms of chain orientation in these model systems.

The observation that coiled-coils are formed in disulfide-bridged peptides in spite of the interchain electrostatic repulsions is indeed intriguing. Positioned appropriately, disulfide bonds can generally make substantial contributions to the stability of coiled-coils (Lehrer, 1978; Holtzer et al., 1986; Engel et al., 1991; Zhou et al., 1993). This increase in stability has been attributed to the decrease in entropy gained during unfolding of the coiled-coil structure (Engel et al., 1991). Molecular modeling studies (Zhou et al., 1992c) have shown that the terminal ends of the coiled-coil are more flexible than the central portion, and this flexibility effectively reduces the stabilizing effect of hydrophobic interactions. However, a disulfide bridge between two terminal ends of each α-helical chain may be expected to facilitate coiled-coil formation by providing an initiation site, as well as an extension, of the hydrophobic core, provided that the interface of the coiled-coil (positions a, d, e, and g of both helices) has a net charge close to zero (Adamson et al., 1993; Zhou et al., 1994). Therefore, the increase in stability may result not only from the contribution of the disulfide bond itself (Zhou et al., 1993) but also from enhanced hydrophobic interactions in one end of the coiled-coil. While it is not inconceivable that the disulfide-bridged coiled-coil may exist in dynamic equilibrium with a disulfide-bridged random-coil structure, it appears that the additional stability from the disulfide bridge is more than enough to override the destabilizing effect of interchain electrostatic repulsions.

Interpretations of the Stability Data. Despite the extensive use of GdnHCl and urea in protein denaturation studies, it is still unclear whether the stability values obtained are dependent on or independent of denaturant (Creighton, 1993). Free energy values calculated from solvent denaturation data can further be complicated by differences in the methods of calculation (Makathadze & Privalov, 1992; Pace, 1986), and this results in the ambiguity in the interpretation of protein stability data. Since in our coiled-coil models our main interest was their relative stabilities, rather than the free energy of unfolding in the absence of denaturant ($\Delta G_u{}^{H_2O}$), we are reporting only the $[denaturant]_{1/2}$ values, from which we calculated the $\Delta\Delta G_u$ values between analogs using the equation proposed by Sali et al (1991): $\Delta\Delta G_u = \{(m_1 + m_2)/2\}\{([denaturant]_{1/2})_2 - ([denaturant]_{1/2})_1\}$. $\Delta\Delta G_u$ is the free energy difference between peptide analogs, and m is the slope term from the equation $\Delta G_u = \Delta G_u{}^{H_2O} - m[denaturant]$.

The GdnHCl denaturation profiles (Figure 5A) showed that P/A had the same $[GdnHCl]_{1/2}$ as P/R (2.6M), giving a $\Delta[GdnHCl]_{1/2}$ value of 0 (Table 2), while AP/A had only a slightly higher $[GdnHCl]_{1/2}$ than AP/R (4.0 versus 3.6 M,

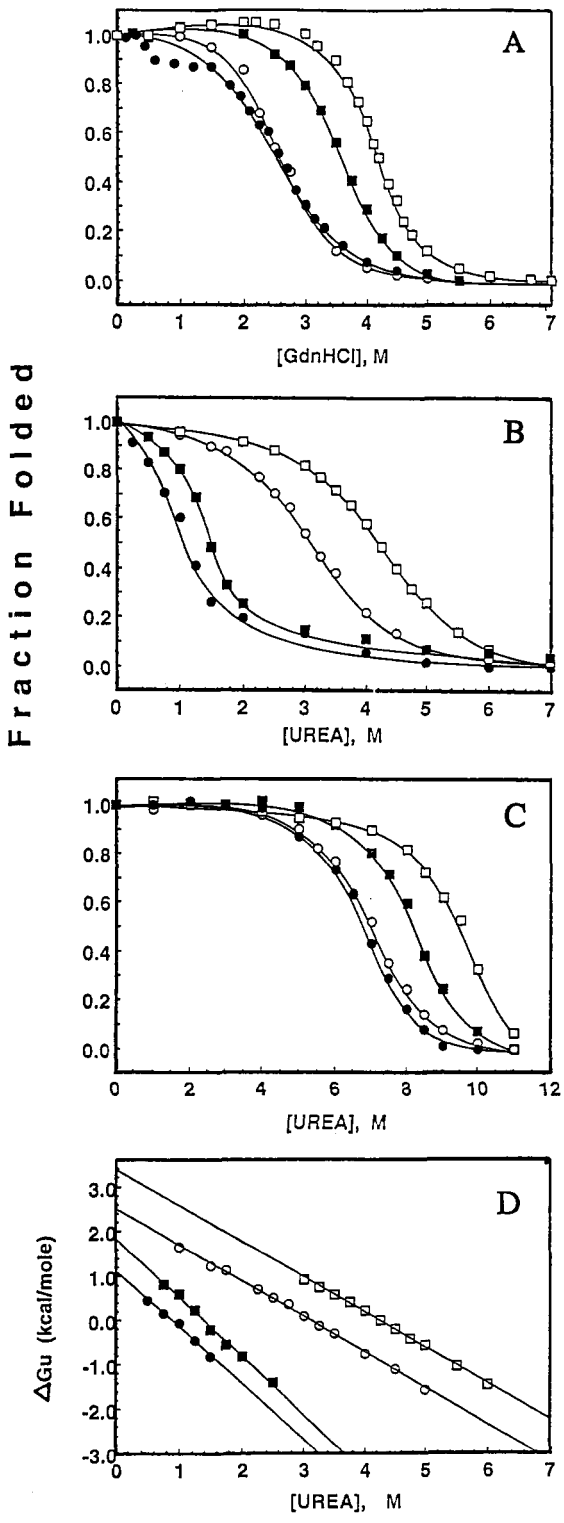

FIGURE 5: Denaturation profiles of coiled-coils in (A) GdnHCl, (B) urea, and (C) urea in the presence of 0.5 M $NaClO_4$/1 M $MgCl_2$. The molar fraction of folded peptide (f_n) was calculated from the equation $f_n = ([\Theta] - [\Theta]_u)/([\Theta]_n - [\Theta]_u)$, where $[\Theta]$ is the observed mean residue ellipticity at 220 nm at any particular denaturant concentration and $[\Theta]_n$ and $[\Theta]_u$ are the mean residue ellipticities of the native (folded) and denatured (unfolded) states. (D) Representative plots of ΔG_u at different urea concentrations for the data displayed in panel B: ○, parallel with interchain electrostatic attraction (P/A); ●, parallel with interchain electrostatic repulsion (P/R); □, antiparallel with interchain electrostatic attraction (AP/A); ■, antiparallel with interchain electrostatic repulsions (AP/R).

respectively, for a $\Delta[\text{GdnHCl}]_{1/2}$ value of 0.4 M; Table 2). These results show that GdnHCl was not very effective in distinguishing the differences in stabilities due to interchain ionic interactions. However, urea denaturation studies showed dramatic differences in stabilities between peptides with different electrostatic interactions. For example, the $[\text{urea}]_{1/2}$ values of coiled-coils with interchain electrostatic attractions (P/A, 3.1 M, and AP/A, 4.2 M) were much higher than those with interchain electrostatic repulsions (P/R, 0.8 M, and AP/R, 1.4 M) (Figure 5B and Table 1), with $\Delta[\text{urea}]_{1/2}$ values of 2.3 (P/A – P/R) and 2.8 M (AP/A – AP/R) (Table 2). Similar interpretations can be obtained from the calculated $\Delta\Delta G_u$ values (Table 2). When electrostatic interactions were masked by conducting urea denaturations in the presence of a salt mixture, the order of stability between analogs was similar to that from GdnHCl denaturations (compare panels A and C of Figure 5 and Table 1). The general increase in $[\text{urea}]_{1/2}$ values was also expected due to salt-induced hydrophobic interactions. Finally, a good inverse correlation ($r = -0.93$) was observed between the $[\text{urea}]_{1/2}$ values and their retention times on a reversed-phase column (Table 1), consistent with the previous suggestion (Zhou et al., 1990) that the more stable coiled-coils are less retained by the hydrophobic matrix.

On the other hand, both GdnHCl and urea can differentiate the effects of the changes in hydrophobic interactions as a result of the difference in the parallel or antiparallel orientation of α-helical chains. For example, the $[\text{GdnHCl}]_{1/2}$ values for P/A and AP/A were 2.6 and 4.0 M, respectively, for a $\Delta[\text{GdnHCl}]_{1/2}$ of 1.4 M (Table 2). Similarly, the $[\text{urea}]_{1/2}$ values were 3.1 and 4.2 M, respectively (Table 1), with a $\Delta[\text{urea}]_{1/2}$ of 1.1 M (Table 2). These results show that when the interchain electrostatic interactions are the same, the antiparallel coiled-coils are consistently more stable than the parallel coiled-coils.

These results are consistent with the well-understood charged nature of the GdnHCl molecule, which, acting like a salt, suppresses interchain electrostatic interactions. The $[\text{GdnHCl}]_{1/2}$ value had been suggested to represent the contributions of hydrophobic interactions to the stability of the protein in the absence of electrostatic interactions (Monera et al., 1993). In contrast, since urea is an uncharged molecule lacking the ionic properties of GdnHCl, it does not suppress the electrostatic interactions and the $[\text{urea}]_{1/2}$ values are therefore indicative of the overall stability of the protein including both electrostatic and hydrophobic interactions. It must be noted, however, that in addition to their relative differences in acting upon the electrostatic interactions, GdnHCl and urea also have similar properties, such as breaking hydrogen bonds that stabilize proteins and binding to the surface of unfolded proteins by hydrogen bonding (Creighton, 1993).

It is also interesting to note that urea denaturation of the two coiled-coils with interchain electrostatic repulsions gave the same slope (m) values, which were steeper than those of the other two coiled-coils with interchain electrostatic attractions, which in turn had the same slopes (Figure 5D). It has been suggested that the m value measures the ability of the denaturant to unfold a protein (Green & Pace, 1974) and that this value is proportional to the number of denaturant molecules interacting at the unfolded sites (Mayo & Baldwin, 1993). It therefore appears that urea is more efficient in unfolding the coiled-coils with interchain electrostatic repulsions than those with attractions. This is not surprising if we consider the denaturation process as an equilibrium between the folded and unfolded states. The interchain electrostatic repulsions, which are not suppressed by the uncharged urea

Table 2: Changes in Free Energies among Coiled-Coil Analogs

parameters	GdnHCl denaturation		urea denaturation	
	$\Delta[\text{GdnHCl}]_{1/2}$[a] (M)	$\Delta\Delta G_{u(\text{GdnHCl})}$[b] (kcal/mol)	$\Delta[\text{urea}]_{1/2}$[c] (M)	$\Delta\Delta G_{u(\text{urea})}$[d] (kcal/mol)
repulsion to attraction				
parallel	0	0	2.3	2.4
antiparallel	0.4	0.6	2.8	3.0
parallel to antiparallel				
attraction	1.4	2.1	1.1	0.9
repulsion	1.0	1.2	0.6	0.8

[a] This represents the change in $[\text{GdnHCl}]_{1/2}$ values when either the interchain electrostatic interactions are changed from repulsion to attraction ($\Delta[\text{GdnHCl}]_{1/2} = [\text{GdnHCl}]_{1/2(\text{attractions})} - [\text{GdnHCl}]_{1/2(\text{repulsions})}$) or the chain alignment is changed from parallel to antiparallel ($\Delta[\text{GdnHCl}]_{1/2} = [\text{GdnHCl}]_{1/2(\text{antiparallel})} - [\text{GdnHCl}]_{1/2(\text{parallel})}$). [b] This represents the difference in the change in free energies when either the interchain electrostatic interactions are changed from repulsion to attraction, $\Delta\Delta G_u = \{(m_{(\text{attractions})} + m_{(\text{repulsions})})/2\}\{[\text{GdnHCl}]_{1/2(\text{attractions})} - [\text{GdnHCl}]_{1/2(\text{repulsions})}\}$ or the chain alignment is changed from parallel to antiparallel, $\Delta\Delta G_u = \{(m_{(\text{antiparallel})} + m_{(\text{parallel})})/2\}\{[\text{GdnHCl}]_{1/2(\text{antiparallel})} - [\text{GdnHCl}]_{1/2(\text{parallel})}\}$. [c] The same as *a*, except that urea is the denaturant. [d] The same as *b*, except that urea is the denaturant.

molecules, would be expected to drive the equilibrium toward the unfolded state, as well as to reduce the probability of refolding once denatured; thus the steeper slope. In contrast, interchain electrostatic attractions would favor the folded state, not only by resisting the unfolding process but also by facilitating the refolding of unfolded chains. Thus, the denaturation process becomes effectively hindered and yields a more gradual slope.

The denaturation of these coiled-coil models was presumed to follow a two-state transition, that is a transition between a coiled-coil and a random coil. This assumption was based on the apparently monophasic denaturation curves (Figure 5), as well as the presence of an isodichroic point in the temperature denaturation curves of these four model peptides (not shown), which has been suggested to be indicative of a two-state transition (Engel et al., 1991). In addition, isolated α-helices are generally thought to be unstable in solution (Dyson et al., 1988; Cohen & Parry, 1990; Creighton, 1993; Thomson et al., 1993). Recently, a thermodynamic characterization of the structural stability of a 56 amino acid residue fragment of GCN4 showed that the unfolding of this coiled-coil was a two-state process in which the helices were only stable when they were in the coiled-coil conformation (Thompson et al., 1993). In addition, denaturation data from some coiled-coils such as the GCN4-p1 peptides (O'Shea et al., 1989a, 1993), the Arc repressor dimer (Bowie & Sauer, 1989), a 32-residue LFBI peptide (De Francesco et al., 1991), and de novo peptides (Zhou et al., 1992a–d, 1993; Zhu et al., 1992, 1993; Engel et al., 1991) have been analyzed on the assumption that the unfolding process follows a two-state equilibrium. Taken together, these results strongly support, but do not unequivocally prove, the existence of a two-state transition for the synthetic coiled-coils used in this study.

However, it cannot be assumed that all coiled-coils follow a two-state denaturation since the transition from coiled-coil to random coil appears to be very fast (Mo et al., 1993) and any possible intermediates may not be easily detectable by conventional CD spectroscopy. In addition, other native coiled-coils appear to exhibit biphasic denaturation curves (Lehrer, 1978; Lehrer et al., 1989; Lehrer & Qian, 1990; Lehrer & Stafford, 1991; Bracken et al., 1988; Greenfield & Hitchcock-DeGregori, 1993), suggesting the presence of folded intermediate states. On this basis, Holtzer and co-workers have proposed the "continuum-of-states" theory (Skolnick & Holtzer, 1986), suggesting that "a broad spectrum of molecular states are allowed" between the coiled-coil form and the unfolded state. This theory had been used to explain the differences in the shapes of temperature denaturation curves of a variety of cross-linked and non-cross-linked tropomyosins (Skolnick & Holtzer, 1986; Holtzer et al., 1990; Bracken et al., 1988). Whether the denaturation proceeds in a two-state or a multi-state fashion will have no effect on the interpretation of our results.

Possible Folding Pathways. On the basis of the present and previous observations (Monera et al., 1993), possible pathways that lead to the formation of the two-stranded parallel and antiparallel coiled-coils are proposed (Figure 6). The high negative molar ellipticity of the reduced starting peptides ($\sim$27 000–28 000 deg cm^2 dmol^{-1}) and the appearance of a single peak in size-exclusion chromatography (not shown) suggest that the reduced peptides predominantly exist in their coiled-coil form. However, it is assumed that under benign conditions the reduced peptides exist in an equilibrium with their random coil forms. This is evident from the observation that, provided the interchain electrostatic interactions are favorable, the major products formed are heterodimers (Figure 3B,E,F). This proposal suggests that the starting reduced homostranded coiled-coils unfold and reform with different strands before disulfide bridge formation.

The proposed pathways also suggest that, under the conditions of air oxidation, the rate of disulfide bridge formation is slower than the rate of dissociation and association of reduced coiled-coils. If we assume that disulfide bridge formation occurs before coiled-coil formation, this pathway would likely be independent of any type of hydrophobic or electrostatic interactions (independent of amino acid sequence) as well as the presence of salts. In other words, if most of the disulfide bridge formation occurred before the starting peptides had a chance to randomize, the homostranded disulfide-bridged products would be expected to predominate in all of these cases. The dependence of product formation on reaction conditions as well as the amino acid sequence (Figure 3B,E,F) suggests that most, but not all, of the disulfide bridge formation occurs after the chains have randomized and reformed (Figure 6). This is consistent with the proposal that α-helices are formed in partially associated peptides before the development of the fully aligned coiled-coil (Greenfield & Hitchcock-DeGregori, 1993) and that electrostatic interactions may dictate the ability of two charged helices to associate (Schuermann et al., 1991; O'Shea et al., 1992, 1993).

Although we have proposed that the majority of the products are formed via these pathways, these are by no means the only possible routes. For example, the formation of a disulfide bond between two random coil monomers is possible even under benign conditions. However, since the proportion of random coil monomers in solution relative to the reduced coiled-coils is presumed to be very small, it follows that this may only be a minor route. In addition, it is also possible that double displacements, in which two dimers meet and exchange chains, cannot be ruled out, though on the basis of the

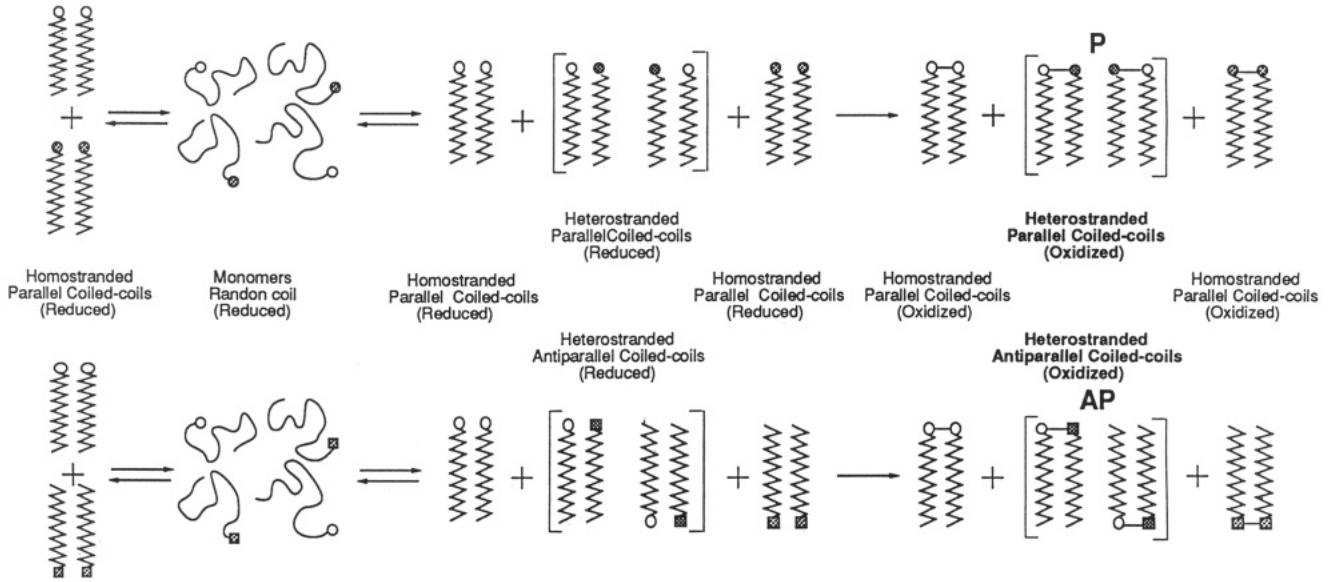

FIGURE 6: Proposed pathways for the formation of disulfide-bridged parallel (P) and antiparallel (AP) heterostranded coiled-coils with interchain electrostatic attractions. Each chain is represented by a zigzag line connected to either a circle (cysteine at position 2) or a square (cysteine at position 33). The open and shaded circles represent two peptides with different sequence. Similarly, the shaded square indicates that the amino acid sequence is different from that of the open circle. In each pathway, two equivalent structures of the heterostranded product are enclosed in brackets to show that, on the basis of a completely random combination of two different peptides, the expected product distribution is a 1:2:1 ratio for the homostranded:heterostanded:homostranded coiled-coils, respectively. This proposal is for what we consider the major pathway and is not to be taken as absolute (see text).

distribution of products this may also be a very minor route. If these alternate routes are responsible for the formation of small amounts of P/R and AP/R (panels C and D, respectively, of Figure 3), in spite of interchain electrostatic repulsions under benign conditions, this would suggest that electrostatic interactions dictate an even higher specificity of parallel or antiparallel orientation than these chromatograms show. For example, it has been estimated that the formation of a heterodimer with interchain electrostatic attractions was preferred 10^5-fold over the homodimeric products with interchain electrostatic repulsions (O'Shea et al., 1993).

Biological Implications. In spite of the apparent higher stability of the antiparallel compared to the parallel coiled-coil, it is intriguing to notice that parallel coiled-coils are more abundant in natural proteins. On the other hand, parallel coiled-coils are not common in globular proteins (Richardson, 1981), except in the dimerization domains in DNA binding proteins (Lamb & McKnight, 1991; Alber, 1992; Adamson et al., 1993). As yet, there are no known examples of proteins that undergo intermolecular association through a two-stranded antiparallel coiled-coil motif, except in multiple helix bundles (Weber & Salemme, 1980; Richardson, 1981; Banner et al., 1987; DeGrado et al., 1989; Cohen & Parry, 1990; Parry et al., 1992; Paliakasis & Kokkinidis, 1992; Lovejoy, 1993). It appears that the parallel orientation is the most simplistic way of bringing two functional domains in register. For example, it is now clear that the parallel alignment of the dimerization domains of GCN4 (O'Shea et al., 1989a, 1991; Saudek et al., 1991; Oas et al., 1990; Ellenberger et al., 1992; Nilges & Brunger, 1991,1993) and GAL4 (Marmorstein et al., 1992) is essential for proper orientation of their DNA binding regions (Landschulz et al., 1988; Lamb & McKnight, 1991; Abel & Maniatis, 1989; Gentz et al., 1989). Similarly, the coiled-coil in the myosin rod has not only a structural role in maintaining the organization of the thick filament but also a role in orienting the myosin head for interactions with actin (Lowey et al., 1969). In both cases, the alignment of the α-helical chains is very precise, most likely controlled by the correct packing of the hydrophobes in the a and d positions of the hydrophobic interface as well as by favorable interchain electrostatic interactions between the charged amino acid residues in the e and g positions. This is consistent with the general observation that residues at positions a, d, g, and e are the most strongly conserved in naturally occuring coiled-coils (Conway & Parry, 1988, 1990).

The results also provide evidence as to why a pair of similar or dissimilar α-helical chains have the disposition to form only parallel or antiparallel coiled-coils and not both. For example, they relate to the suggestion (Conway & Parry, 1990) that the charged residues in positions e and g of fibrous proteins have a common disposition to always specify a parallel, in-register alignment of their α-helical chains. This is not surprising because an antiparallel orientation of these chains would likely result in coiled-coil destabilization by interchain electrostatic repulsions (Parry, 1982). In natural or *de novo* designed coiled-coils the interacting α-helices must be charged differently to allow stabilizing ionic interactions (Cohen & Parry, 1990). Similarly, the opposite charges of the Fos and Jun chains specify and stabilize heterodimeric coiled-coil formation, since both Fos–Fos and Jun–Jun homodimers would be destabilized by electrostatic repulsions of acidic and of basic residues, respectively (Schuermann et al., 1991; O'Shea et al., 1989b, 1991, 1992, 1993).

This study demonstrates that interchain electrostatic interactions control the orientation of α-helical chains in coiled-coils. This information is of fundamental importance in the *de novo* design of proteins containing interacting α-helices as well as in altering the structure and function of naturally occurring coiled-coil proteins.

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