Design and Characterization of an Intramolecular Antiparallel Coiled Coil Peptide†

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ABSTRACT: A 56-residue polypeptide was designed to fold into a stable intramolecular antiparallel coiled coil, referred to as a coiled coil stem loop. The antiparallel orientation of the α-helices was dictated by the alignment of hydrophobic and ionic residues in the heptad repeat sequence $(a, b, c, d, e, f, g)_n$. The hydrophobic core at the coiled coil interface was occupied by leucine and valine residues in heptad positions d and a′ and positions a and d′, respectively. The interface border positions e and g were occupied by glutamic acid in the amino-terminal helix and lysine residues in the carboxy-terminal helix. A loop segment connecting the α-helices began and ended with the helix-breaking residues glycine and proline. Alanine and serine residues were placed on the exposed b, c, and f positions of both helices to increase the helical propensity and solubility of the peptide, respectively. Several lines of evidence argued that the synthetic peptide made with this design folded into a stable monomeric coiled coil stem loop conformation: (1) the peptide was highly soluble in 150 mM sodium chloride and 50 mM sodium phosphate, pH 7.4; (2) the circular dichroism spectrum was α-helical but with relative ellipticity minima at 222 and 208 nm characteristic of a coiled coil structure; (3) the peptide exhibited an α-helical content near 80%, which was independent of peptide concentration and unchanged in the presence of trifluoroethanol; (4) size exclusion chromatography and sedimentation equilibrium ultracentrifuge measurements confirmed that the peptide was monomeric in aqueous solution; (5) the peptide exhibited high helical content over a wide pH range; (6) the apparent T_m for unfolding the α-helical structure was greater than 65 °C, and 3.0 M urea was required to reduce the helical structure by 50%; (7) a disulfide bond was readily formed in the monomer between the amino- and carboxy-terminal cysteine residues, confirming the antiparallel orientation of the helices; and (8) the peptide competed with fibrinogen for the GPIIbIIIa receptor indicating that the RGD residues present in the loop sequence were available for binding. This work establishes that an antiparallel alignment of α-helices can be achieved by designing specific hydrophobic and ionic interactions within the coiled coil. The prototype coiled coil peptide represents a sequence-simplified scaffold into which residues from α-helices and loops of native proteins can be inserted to form conformationally constrained mimetic recognition molecules.

Recognition of folded macromolecules is a recurrent theme which underlies essentially all biological processes. Mimicking binding sites of macromolecules can provide recognition molecules of use in biotechnology, for therapeutics, diagnostics, and separation. Biological macromolecules such as proteins can be thought of as composites of conformational frameworks, or scaffolds, in which a limited number of recognition elements are presented on the surface. Hence, *de novo* construction of idealized scaffolds, in which recognition elements from naturally occurring proteins and peptides are embedded, can provide a generalized approach to mimetics and antagonist design.

The α-helix is a structural motif of proteins which often presents recognition sequences. For example, proteins such as interleukins 2 and 4 and growth hormone bind to their receptors through residues arrayed predominantly on the surfaces of α-helices (Bazan, 1992; McKay, 1992; Redfield *et al.*, 1991; De Vos *et al.*, 1992). And, the papillomavirus E2 and λ repressor proteins have helical recognition sites which directly bind to DNA base pairs (Hedge *et al.*, 1992; Jordan, 1988). For these types of interactions, idealized helical frameworks would be useful for presentation of recognition surfaces in mimetics and antagonists. Yet, no fully satisfactory α-helical frameworks are available for mimetics discovery. Unfortunately, typical single-stranded polypeptide chains generally do not form stable α-helices in aqueous solution and require the additional stabilization of lower temperature or less polar solvents (Brown & Klee, 1971; Bierzynski *et al.*, 1982). Hence, single-stranded peptides are not useful in mimicking helical structures for most biotechnological applications.

One potentially useful structure for α-helical mimicry which may overcome the limitation of conformational instability is the coiled coil. The coiled coil structure is widely found in both fibrous and globular proteins where it functions as an integral structural component, presenting residues involved in recognition surfaces or dimerization domains. The coiled coil conformation is stabilized by the interactions between α-helices: two right-handed α-helices wind around one another in a left-handed supercoil (Cohen & Parry, 1990). Supercoiling optimizes the interchain packing of hydrophobic and ionic residues which are responsible for the stability and orientation of the coiled coil (Skolnick & Holtzer, 1985; Mo *et al.*, 1990; O'Shea *et al.*, 1991). In general, the coiled coil structure allows polypeptides to form extremely stable α-helices and is tolerant to changes in sequence as long as the hydrophobic and hydrophilic heptad repeat is conserved. These features make the coiled coil structure an ideal candidate for use in α-helical mimicry.

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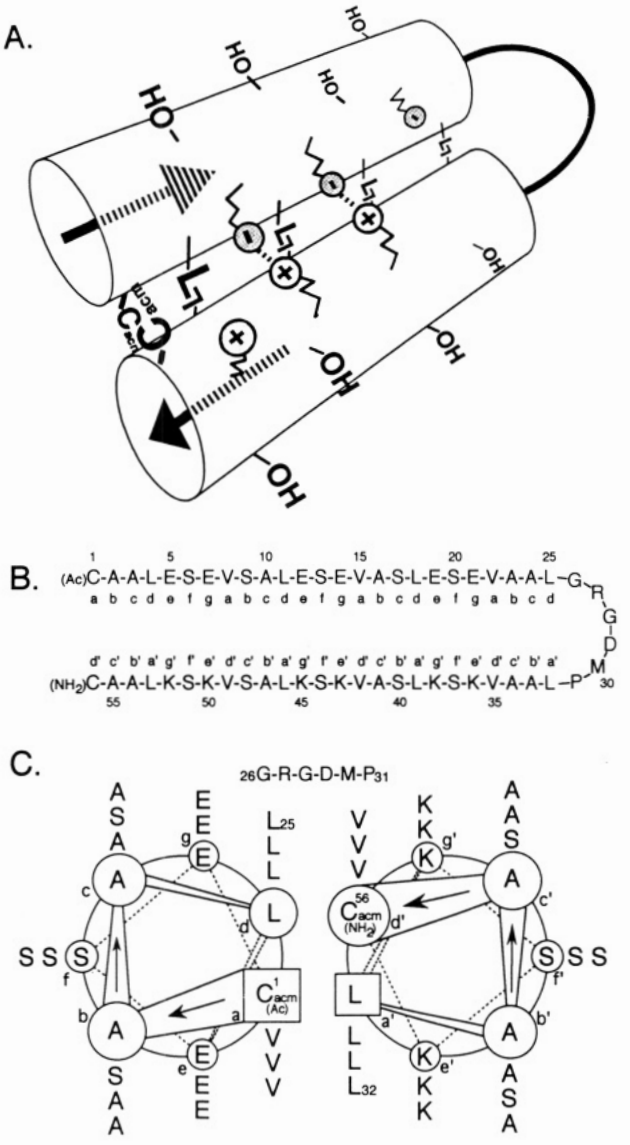

FIGURE 1: (A) Proposed structure of the intramolecular antiparallel coiled coil stem loop (CCSL) peptide. For simplicity the supercoiled helices are depicted here as straight cylinders. The side chains of the leucine, glutamic acid, lysine, and serine residues are represented by L, ⊖, ⊕, and OH, respectively. The amino- and carboxy-terminal (acetamidomethyl)cysteine residues are represented by C_{acm}. (B) The 56-amino acid residue sequence of the CCSL peptide. The positions of the heptad repeat are denoted a, b, c, d, e, f, and g for residues 1–25 and a′, b′, c′, d′, e′, f′, and g′ for residues 32–56. The two helices are joined by a six-residue loop occupying positions 26–31. (C) Helical wheel representation of the CCSL peptide viewed from the amino and the carboxy terminus. Leucine residues at position d in the amino-terminal α-helix interact across the interface with residues at position a′ in the carboxy-terminal helix.

We have applied the basic principles involved in the formation of a stable coiled coil to design a peptide which folds into an intramolecular antiparallel coiled coil structure as shown schematically in Figure 1A, referred to here as a coiled coil stem loop (CCSL).[1] To stabilize the structure of the CCSL peptide, hydrophobic amino acids leucine and valine have been selectively incorporated in the heptad repeat positions d and a′ and positions a and d′, respectively. To direct the folding of an antiparallel orientation of α-helics, the ionic residues glutamic acid and lysine were incorporated into heptad positions e and g and positions e′ and g′, respectively. A six-residue loop sequence which connects the α-helices began and ended with the helix-terminating and-initiating residues glycine and proline, respectively. Residues not involved in the coiled coil interface (heptad positions b, c, and f) were occupied by serine and alanine. Figure 1B shows the complete linear sequence of the CCSL peptide which was designed and synthesized in this study. This peptide has been extensively characterized by circular dichroism, size exclusion chromatography, and sedimentation equilibrium measurements and found to have a stable monomeric coiled coil structure in aqueous solution. Therefore, this peptide represents a sequence-simplified scaffold into which residues from surfaces of α-helices and loop segments of native proteins or peptides may be inserted to form conformationally constrained mimetic recognition molecules. A preliminary report of this work has been presented at the 13th American Peptide Symposium, Edmonton, Alberta, Canada, in June 1993 (Myszka & Chaiken, 1994).

EXPERIMENTAL PROCEDURES

Materials. TFA, TFE, and acetonitrile were HPLC grade and were purchased from Baker (Philipsburg, NJ). HPLC and FPLC columns were from Vydac (Hesperia, CA), Millipore Co. (Bedford, MA), and Pharmacia (Piscataway, NJ). Gel filtration molecular mass markers and anti-biotin antibody conjugated to alkaline phosphatase were from Sigma Chemical Company (St. Louis, MO). All amino acid derivatives and resins used for peptide synthesis were made by American Peptide Company (Sunnyvale, CA). The *p*-nitrophenyl phosphate was from Bio-Rad (Richmond, CA). Fibrinogen was purchased from Cal Biochem (La Jolla, CA) and biotinylated using sulfo-*N*-hydroxysuccinimidobiotin from Pierce (Pockford, IL). Linbro EIA II plus microtiter plates were from Flow Lab (McLean, VA). GPIIbIIIa was provided by Kyung Johanson at SmithKline Beecham. The 42-residue homodimer coiled coil peptide $(ESKVSSL)_6$ was prepared previously (Graddis *et al.*, 1993).

Peptide Synthesis and Purification. The CCSL peptide was synthesized and purified by the American Peptide Company Inc. (Sunnyvale, CA). Merrifield solid-phase peptide synthesis was started with *p*-methylbenzhydrylamine resin and carried out using the standard *tert*-butyloxycarbonyl (*t*-Boc) strategy (Matsueda & Stewart, 1981) on a Beckman 990 automated peptide synthesizer. Cleavage from the resin and removal of all protecting groups was accomplished by using "low–high" HF cleavage (Tam *et al.*, 1983). Peptide resin (4 g) was treated by HF/DMS/*p*-cresol for 110 min at 0 °C. The HF and DMS were removed in vacuo at 0 °C, and then the vessel was recharged with 40 mL of anhydrous HF for 60 min at −5 °C. The HF was removed under reduced pressure, and the resin was washed with cold anhydrous ether. The peptide was extracted with water and lyophilized. The crude powder (2.2 g) was dissolved in 100 mL of 11% acetonitrile in water and purified by preparative reverse-phase HPLC using δ-pack C18 (Millipore Co.).

Purity, Amino Acid Analysis, and Mass Spectrometry. Peptide purity was verified by reverse-phase HPLC using a C18 column (Vydac, 4.6 mm × 25 cm). Elution was done with a linear gradient of 30 to 60% acetonitrile containing

[1] Abbreviations: CCSL, coiled coil stem loop; $(ESKVSSL)_6$, 42-residue homodimer coiled coil peptide; CD, circular dichroism; HPLC, high-performance liquid chromatography; FPLC, fast-protein liquid chromatography; PBS, phosphate-buffered saline; Acm, acetamidomethyl; TFA, trifluoroacetic acid; TFE, trifluoroethanol; CNBr, cyanogen bromide; HF, hydrogen fluoride; T_m, temperature required to produce 50% of the folded structure; IC_{50}, concentration for 50% inhibition; M_r, molecular mass; Da, dalton; ΔG_u, Gibbs free energy in the absence of denaturant.

0.1% TFA at a flow rate of 1.5 mL/min, for 20 min. Quantitative amino acid analysis after acid hydrolysis was carried out on a Beckman 6300 amino acid analyzer. Fast atom bombardment analysis was done on M-Scan's VG Analytical ZAB 2-SE high-field mass spectrometer operating at *V*acc = 8 kV. A cesium ion gun was used to generate ions for the acquired mass spectra, which were recorded using a PDP 11-250J data system. Mass calibration was performed using cesium iodide.

Cyanogen Bromide Cleavage. The single methionine present in the CCSL peptide at position 30 was used as a cleavage site for CNBr. The digestion was carried out by incubating 0.2 μmol of the peptide in 100 μL of 70% formic acid and 20 μmol of CNBr for 16 h at room temperature in the dark (Spande *et al.*, 1970). The reaction mixture was lyophilized four times, and the resulting CNBr-cleaved CCSL peptide was diluted to a concentration of 250 μM in 150 mM sodium chloride and 20 mM phosphate, pH 7.4.

Gel Filtration Chromatography. Gel filtration chromatography was performed on a 30/16 Superdex-75 FPLC column (Pharmacia) in 150 mM sodium chloride and 20 mM phosphate (PBS), pH 7.4, at a flow rate of 0.5 mL/min at 22 °C. The elution profiles were monitored at an absorbance wavelength of 215 nm. The CCSL and $(ESKVSSL)_6$ peptides were loaded on the column in a 100-μL injection at concentrations of 30, 3, and 0.3 μM. Standard globular proteins were loaded at a concentration of 0.5 mg/mL and included bovine serum albumin, carbonic anhydrase, cytochrome c, and aprotinin (M_r = 66, 29, 12.4, and 6.5 kDa, respectively). For gel filtration experiments performed in 50% trifluoroethanol (TFE), the PBS was diluted with TFE (1:1, v/v).

Analytical Sedimentation Equilibrium. Analytical sedimentation equilibrium studies were performed on a Beckman Optima XL-A analytical ultracentrifuge. A 20 μM concentration of the CCSL peptide in PBS was centrifuged to equilibrium at 40 000 rpm and 4 °C. The absorbance across the sample cell was determined at 235 nm versus the reference cell. The data was analyzed as described by Brooks *et al.* (1994) using the partial specific volumes for the amino acids reported by Cohn and Edsall (1943).

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectra were measured with a Jasco J-500C spectropolarimeter attached to a Lauda (Model RMS) water bath used to control the cell temperature. The spectropolarimeter was interfaced to a Macintosh computer for data collection and manipulation. All spectra were measured at 270–185 nm using a 0.1-cm cell, a 1-nm bandwidth, and a 1-s time constant. Four scans were averaged for each spectrum and then corrected for solvent contributions. Measured rotations were converted to mean molar residue ellipticity [Θ] (deg cm^2 $dmol^{-1}$) (Schmid, 1989). All spectra were recorded with a cell temperature of 25 °C unless otherwise stated. The percentage of α-helical content was estimated directly from the molar residue ellipticity at 222 nm using the method described by Chen *et al.* (1974). All concentrations of stock peptide solutions were determined by amino acid analysis.

To test the effect of the helix-inducing solvent trifluoroethanol (TFE) on α-helical content, CD spectra were recorded for a solution of 20 μM CCSL peptide in PBS alone and in a 50% TFE/PBS buffer. The latter was generated by diluting the PBS with TFE (1:1, v/v).

To determine the effect of peptide concentration and CNBr cleavage on α-helical content, CD spectra were recorded for the intact and CNBr-cleaved CCSL peptides at concentrations from 0.2 to 500 μM in PBS using a 0.1-cm cell. Limitations in the signal strength made it difficult to go to lower concentrations. The use of protein solutions as dilute as 0.6 μg/mL raised concerns about protein sticking to the walls of the sample tubes and CD cells. The danger is that adsorbed material may be an appreciable fraction of the total, thus reducing the effective concentration in the cell. This was guarded against by rinsing the cell with the solution before loading the actual sample (Isom *et al.*, 1984).

To test the effect of salt concentration on the α-helical content, CD spectra were recorded for a 20 μM concentration of the CCSL peptide in 20 mM phosphate buffer, pH 7.4, alone and in buffer containing 0.15 M and 1 M sodium chloride.

To determine the effect of pH on α-helical content, CD spectra of the CCSL peptide at a concentration of 20 μM were recorded from pH 0.6 to 12.5. Stock peptide solutions were prepared with 150 mM sodium chloride in dilute phosphoric acid, 20 mM phosphate, and dilute sodium hydroxide. The desired pH was generated by mixing the appropriate buffered peptide solutions. The samples were incubated at the desired pH for 1 h before the CD spectra were taken.

To record the thermal denaturation profile, CD spectra were measured on a 20 μM concentration of the CCSL peptide in PBS over a temperature range of 5–80 °C. The sample cell temperature was raised in 5 °C increments, and the sample was incubated for 15 min at each temperature before the CD spectra were taken.

The urea denaturation studies were carried out by preparing CCSL peptide stock solutions at a concentration of 20 μM in PBS alone and with 6 M urea in PBS. Different ratios of the PBS and 6 M urea solutions were mixed to give the appropriate final urea concentrations for the CD measurements. Mixed samples were incubated for 1 h before analysis.

Disulfide Bond Formation within the CCSL Peptide. The acetamidomethyl blocking groups were removed from the side chains of the amino- and carboxy-terminal cysteine residues of the CCSL peptide by utilizing iodine (Kamber, 1971). One milligram of peptide was dissolved in 1 mL of 50% acetic acid at room temperature. The sample was stirred while 200 μL of a solution of iodine (1 mg) in 100% acetic acid was added over 1 h. The red solution was cooled on ice and 1 M aqueous sodium thiosulfate was added until the red color was removed, followed by an additional 50% excess of thiosulfate. The disulfide product was desalted by gel filtration chromatography through Sephadex G-25. Reduction of the disulfide bond within the CCSL peptide was accomplished with 5 mM dithiothreitol at room temperature for 30 min. To reform the disulfide bond, the dithiothreitol was removed by desalting and air was bubbled through the sample overnight. Reverse-phase HPLC of the Acm-blocked, reduced, and oxidized CCSL peptides was performed on a Vydac C18 column. Samples were eluted with a linear gradient of 0 to 70% acetonitrile containing 0.1% TFA in 20 min.

RGD Binding Assay to GPIIbIIIa. The binding of the CCSL peptide and the GRGDMP peptide to purified GPIIbIIIa was determined by the ability to compete for binding of biotinylated fibrinogen in the solid-phase receptor binding assay described by Smith *et al.* (1990). GPIIbIIIa was coated onto the activated microtiter plates at a concentration of 1 μg/mL (0.1 μg/well) by incubation overnight at 4 °C. The plates were washed and prepared for use. Biotinylated fibrinogen was added to the sample wells followed by the CCSL, control GRGDMP, and standard RGD peptides at the desired starting concentration in 10 mM phosphate, pH 7.5. Plates were incubated for 3 h at 30 °C and washed, and

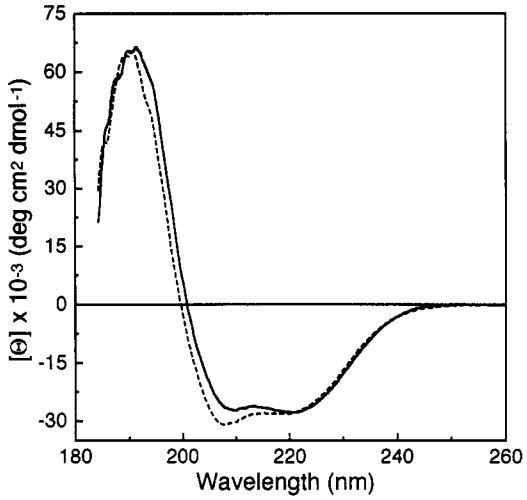

FIGURE 2: Circular dichroism spectra of 20 μM CCSL peptide in the presence (dashed line) and absence (solid line) of the α-helix-inducing solvent trifluoroethanol (TFE). The buffer was 150 mM sodium chloride and 20 mM phosphate (PBS), pH 7.4, and the spectra were taken at 25 °C. For the sample containing TFE, the PBS buffer was diluted with TFE (1:1, v/v).

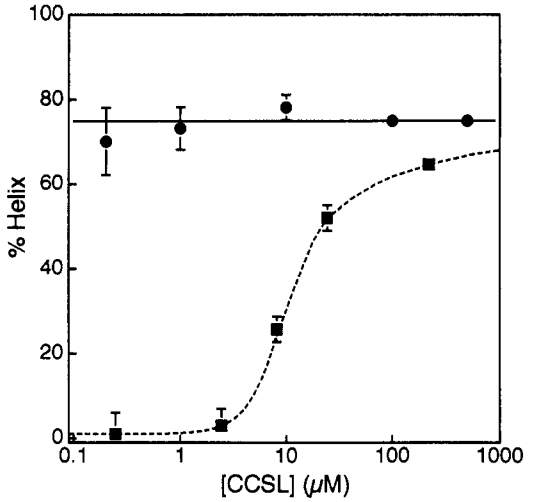

FIGURE 3: Effect of peptide concentration and CNBr cleavage on α-helical content of the CCSL peptide. The percent α-helix as determined from the ellipticity at 222 nm is reported versus the concentration of CCSL (circles, solid line) and CNBr-cleaved CCSL (squares, dashed line) from 0.2 to 500 μM in PBS.

the anti-biotin antibody conjugated to alkaline phosphatase was added and incubated for 1 h at 30 °C. The plates were washed and incubated with the *p*-nitrophenyl phosphate substrate for 10 min with shaking. The reaction was stopped with 0.4 N sodium hydroxide, and the results were read at 405 nm on a Molecular Devices Thermomax microplate reader (Menlo Park, CA). IC_{50} values were determined from a "four-parameter fit" using the Softmax software program from Molecular Devices.

RESULTS

Purity, Composition, Mass Spectrometry, and Solubility of the CCSL Peptide. Analytical reverse-phase HPLC of the purified CCSL peptide revealed a single peak showing peptide homogeneity of greater than 95%. Quantitative amino acid analysis confirmed the intended composition for the CCSL peptide: Asp, 1.1 (1); Ser, 9.8 (10); Glu, 6.1 (6); Pro, 1.3 (1); Gly, 2.1 (2); Ala, 12.1 (12); Val, 5.6 (6); Met, 0.9 (1); Leu, 8.3 (8); Lys, 5.6 (6); Arg, 1.1 (1); Cys, not determined (2). The CCSL peptide gave a positive ion FABMS spectrum with an intense possible $(M + H)^+$ pseudo molecular ion cluster centered at m/z 5790 (calculated from sequence M_r = 5789.4 Da). The CCSL peptide was highly soluble in both water and phosphate-buffered saline (PBS) solutions. Concentrations as high as 8 mM have been achieved.

α-Helical Content of the CCSL Peptide. In PBS, the CCSL peptide exhibited a circular dichroism spectrum indicating high α-helical content, with large molar ellipticity minima values at 222 and 208 nm and a maximum ellipticity around 193 nm (Figure 2). The α-helical content of the 56-residue polypeptide, calculated from the observed molar ellipticity at 222 nm (–28 000 deg cm^2 dmol^{-1}), was estimated to be about 75% (Chen *et al.*, 1974). The ellipticity minimum at 222 nm was unchanged upon adding the helix-inducing solvent trifluoroethanol (TFE, 50%) (Figure 2). The inability of TFE to increase the helical content of the CCSL peptide indicates that under the conditions used (pH 7.4, 25 °C) the peptide is near its maximum α-helical formation. TFE is considered to be a noninteracting solvent that induces helicity in a single-chain, potentially α-helical polypeptide (Nelson & Kallenbach, 1986). The less polar solvent induces and stabilizes α-helical structure in a peptide but not indiscriminately (Hodges *et al.*, 1990). That is, regions that have a high tendency to adopt an α-helical conformation become more α-helical in TFE, whereas regions that are helix-disfavoring are not induced to become α-helical (Nelson & Kallenbach, 1986, 1989; Hodges *et al.*, 1990). The loop sequence and the previously reported flexibility of the amino- and carboxy-terminal ends of the coiled coil (Zhou *et al.*, 1992a) are most likely what reduced the α-helical content from 100%.

The addition of TFE to the aqueous buffer caused a shift and increase in the ellipticity minimum around 208 nm (Figure 2). This excitation band polarizes parallel to the helix axis and is sensitive to whether the α-helix is single-stranded or is an interacting helix as in the case of two-stranded coiled coils (Zhou *et al.*, 1992a). An increase and blue shift in ellipticity at 208 nm has been shown to correspond to conversion of an α-helical coiled coil structure to a rigid single-stranded α-helix (Cooper & Woody, 1990; Zhou *et al.*, 1992a). For the CCSL peptide at 25 °C, the magnitude of the molar ellipticity ratio at 222 and 208 nm ($[\Theta]_{222}/[\Theta]_{208}$) was 1.02 in PBS and was reduced to 0.90 in 50% TFE. Previous studies on parallel coiled coil peptides report a maximum $[\Theta]_{222}/[\Theta]_{208}$ ratio of 1.03 for a highly coiled two-stranded α-helical coiled coil in aqueous buffer solutions (Lau *et al.*, 1984; Hodges *et al.*, 1988, 1990; Zhou *et al.*, 1992b) and about 0.86 for a single-stranded α-helix in the presence of TFE (Zhou *et al.*, 1992a). The high $[\Theta]_{222}/[\Theta]_{208}$ ratio suggests that the peptide assumes a coiled coil structure in aqueous buffer. In the presence of 50% TFE, the decreased $[\Theta]_{222}/[\Theta]_{208}$ ratio along with the shift in ellipticity minima around 208 nm are consistent with the conversion of a coiled coil to a single-stranded α-helix.

CD spectra were obtained at varying CCSL peptide concentrations. Within experimental error, the CCSL peptide showed no significant change in α-helical content, as determined from the mean residue ellipticity minimum at 222 nm (Figure 3), and no significant difference in the $[\Theta]_{222}/[\Theta]_{208}$ ratio over a 2500-fold peptide concentration range (500–0.2 μM) (data not shown). This concentration independence suggests that the CCSL peptide folds into an intramolecular coiled coil. As a control for this experiment, the concentration dependence of helicity was also measured for a solution of CNBr-cleaved CCSL. The CCSL peptide contains a single methionine in the loop segment connecting the amino- and carboxy-terminal α-helices (Figure 1B). CNBr digestion at

this methionine resulted in cleavage of the CCSL peptide into two peptides. In contrast to the results for the intact CCSL peptide, the CNBr-cleaved peptide showed a strong concentration dependence on α-helical content (Figure 3). At the highest peptide concentration, the observed α-helical content of the CNBr-cleaved CCSL peptide approached the level observed for intact CCSL peptide. However, below micromolar concentration the α-helical signal observed for the CNBr-cleaved peptide was completely lost. Peptides with α-helical structures that are dependent on dimerization or oligomerization show a loss of α-helical content as the peptide concentration is decreased (Zhou *et al.*, 1992b; Kaumaya *et al.*, 1990). These results suggest that the CNBr-cleaved CCSL peptide forms an intermolecular coiled coil and support the conclusion that by contrast the intact CCSL peptide forms an intramolecular coiled coil.

Evidence that the CCSL Peptide is a Monomer in Solution. Gel filtration chromatographic analysis for size was performed on the CCSL peptide. The results were compared with data for $(ESKVSSL)_6$, a 42-residue polypeptide previously obtained and shown to form an intermolecular homodimer coiled coil (Graddis *et al.*, 1993). The CCSL peptide eluted from the gel filtration column as a single peak and at the same position whether the peptide was loaded at a concentration of 30, 3, or 0.3 μM (Figure 4, panels A, B, and C, respectively). With the column calibrated for standard globular proteins, the elution volume of the CCSL peptide corresponded to an apparent molecular mass of 6500 Da. This value is close to the calculated monomeric molecular mass of 5789 Da. The slightly larger size determined by gel filtration is consistent with results seen for other coiled coil peptides. In benign medium, α-helical coiled coils are rod-like in shape and elute more rapidly from a gel filtration column than a globular molecule of identical molecular mass (Hodges *et al.*, 1981). Elution of the CCSL peptide as a single peak, at an apparent molecular mass of 6500 Da, and independent of starting peptide concentration collectively provide evidence that it is a monomer in solution.

In contrast to the elution profiles of the CCSL peptide, the $(ESKVSSL)_6$ peptide eluted from the gel filtration column as two peaks, and the proportions of the peaks changed as the concentration of peptide was lowered (Figure 4A,B,C). The faster eluting peak predominated when the peptide was loaded at a concentration of 30 μM. However, as the peptide starting concentration was decreased to 0.3 μM, the amount of this peak decreased and the amount of the slower eluting peak increased to become the dominant peak. These observations suggest that the faster eluting peak, which corresponds in elution position to an apparent molecular mass of 10 000 Da, is a dimer of the 42-residue $(ESKVSSL)_6$ peptide. The calculated molecular mass of a $(ESKVSSL)_6$ dimer is 8770 Da. The slower eluting peak apparently represents the monomeric form of the peptide, which is in equilibrium with the dimeric state and therefore increases proportionately versus the dimer peak as the peptide concentration is lowered. This behavior of the $(ESKVSSL)_6$ peptide at different concentrations during gel filtration is consistent with the model that the peptide forms a noncovalent dimer in solution. In contrast, from the elution position and concentration independence of the CCSL peptide under identical conditions, the CCSL peptide behaves as a monomer.

Both the CCSL and $(ESKVSSL)_6$ peptides eluted as single peaks from the gel filtration column with an elution buffer containing 50% (v/v) of the helix-inducing solvent TFE (Figure 4D). The $(ESKVSSL)_6$ peptide eluted in the volume seen for

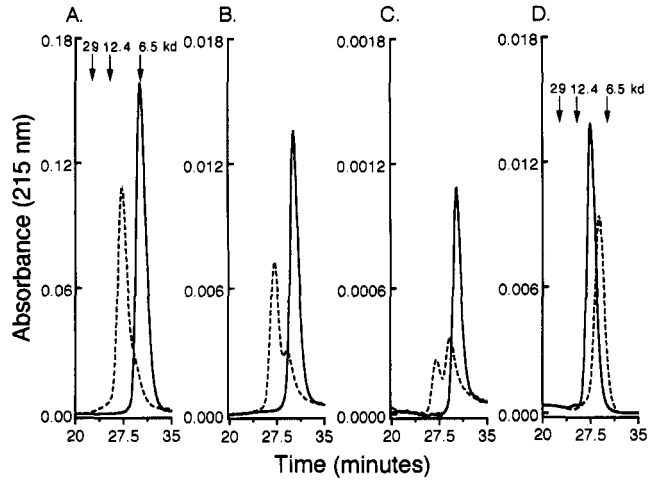

FIGURE 4: Gel filtration chromatography elution profiles for the CCSL peptide (solid lines) and the $(ESKVSSL)_6$ peptide (dashed lines) loaded at concentrations of 30 (A), 3 (B), and 0.3 μM (C), respectively. The Superdex-75 column was run at 22 °C in 150 mM sodium chloride and 20 mM phosphate, pH 7.4, at a flow rate of 0.5 mL/min, and column effluent was monitored at an absorbance of 215 nm. Arrows indicate the positions of the globular protein standards with molecular masses of 29, 12.4, and 6.5 kDa. (D) Elution profiles for the two peptides loaded at a concentration of 3 μM and run in PBS containing 50% TFE (1:1, v/v).

the monomeric peptide eluted in PBS (Figure 4D). There was no evidence of a dimeric species for $(ESKVSSL)_6$ under these conditions, suggesting that the TFE stabilized the peptide into a single-stranded form. In TFE, the CCSL peptide eluted at a position corresponding to an apparent molecular mass of 11 000 Da. This value was much larger than the apparent molecular mass determined for this peptide in PBS (6500 Da). These results suggest that TFE stabilizes the CCSL peptide in an extended single-stranded α-helical conformation, which appears larger by gel filtration due to the large hydrodynamic volume caused by its elongated shape. This result is confirmed by the observation that, in TFE, the CCSL peptide elutes faster than the $(ESKVSSL)_6$ peptide, which is consistent with it being an extended 56-residue peptide versus the 42-residue $(ESKVSSL)_6$ peptide. Together the gel filtration experiments performed in PBS and 50% TFE argue that in PBS the CCSL peptide folds into a compact monomeric structure.

As an added confirmation that the CCSL peptide was monomeric in aqueous solution, analytical sedimentation equilibrium measurements were performed on the peptide in PBS (see Experimental Procedures). The equilibrium data were fit to a monomer with a molecular mass corresponding to 5900 Da and showed no evidence for dimerization.

Salt and pH Effects on the Structure of the CCSL Peptide. The CCSL peptide dissolved in 20 mM phosphate, pH 7.4, showed a 2 and 5% increase, respectively, in α-helical content upon addition of 150 mM and 1 M sodium chloride. This increased helical content with increasing ionic strength is consistent with data for other coiled coil peptides and can be explained by the increased strength of the hydrophobic interactions as the polarity of the medium is increased (Lau *et al.*, 1984; Mo *et al.*, 1990).

CD spectra were recorded for the CCSL peptide at a variety of pHs to analyze the effect on α-helical content and coiled coil structure (Figure 5). The pH profile obtained for the α-helical content showed an asymmetry in going from pH 0.6 to 12.5. The α-helical content abruptly decreased from pH 9.3 to 12.5. This latter transition may be caused by the removal

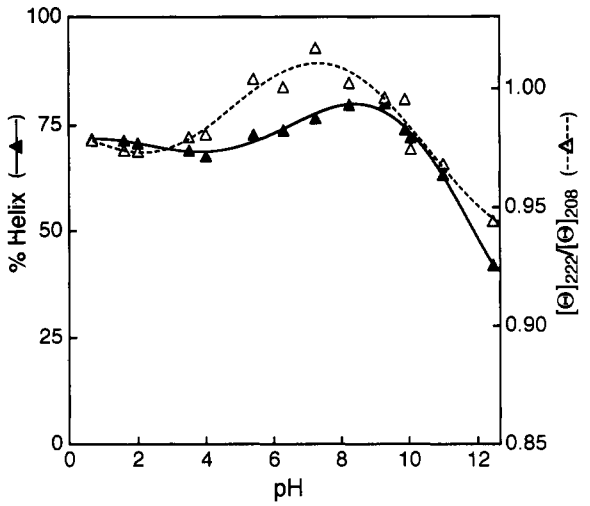

FIGURE 5: pH dependence on α-helicity and coiled coil structure of the CCSL peptide: Percent helix as determined from the ellipticity at 222 nm plotted versus pH (solid line, closed triangles) and corresponding $[\Theta]_{222}/[\Theta]_{208}$ ratio plotted versus pH (dashed line, open triangles).

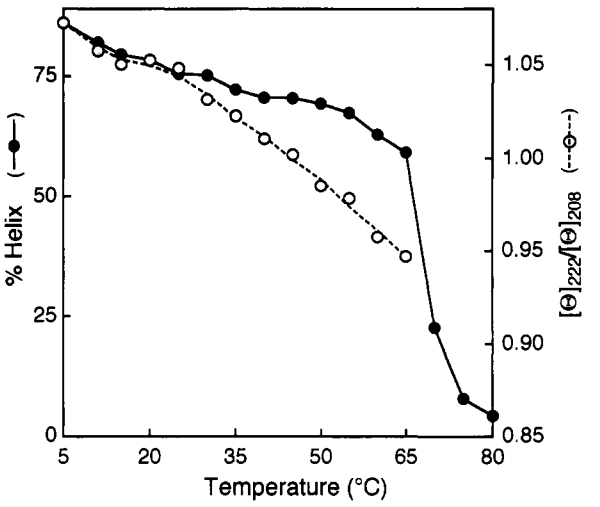

FIGURE 6: Thermal melting profile of the CCSL peptide: Percent helix versus cell temperature (solid line, closed circles) and corresponding $[\Theta]_{222}/[\Theta]_{208}$ ratio versus temperature (dashed line, open circles).

of favorable ion pair and charge–dipole interactions upon deprotonation of the lysine residues. In contrast, the α-helical content decreased less from the maximum at pH 9.3 to 4 and appeared to increase slightly at lower pH. The relatively low sensitivity of helix content to decreasing pH in the pH 8–2 range is not consistent with the expectation that eliminating charge effects (salt bridges and ion–dipole interactions) by protonation of acidic side chains would result in decreased stability of the helix (Chakrabartty *et al.*, 1989). However, an increase in stability at low pH has been reported for a number of natural and model coiled coil peptides (Lowery, 1965; Lau *et al.*, 1984; Hodges *et al.*, 1988; Zhou *et al* ., 1992c; Huyghues-Despointes *et al.*, 1993). It is thought that the free energy of interhelix interaction is predominantly hydrophobic and independent of pH (Skolnick & Holtzer, 1985). However, several other factors which are pH dependent may contribute to the stability of the individual α-helical segments. These include removal of unfavorable short-ranged charge–charge interactions, the effect of unfavorable charge–helix dipole interactions, the existence of singly charged hydrogen bonds at low pH between glutamate and lysine (Marqusee & Baldwin, 1987), changes in helix propensity of glutamate and aspartate (Skolnick & Holtzer, 1985; Huyghues-Despointes *et al.*, 1993), and the increase in hydrophobic interaction of apolar elements of glutamate upon protonation.

As with α-helical content, supercoiling of the CCSL peptide as determined by the $[\Theta]_{222}/[\Theta]_{208}$ ratio also appeared to be sensitive to pH, exhibiting a maximum at pH 7 (Figure 5). Interestingly, the pH optimum estimated from the $[\Theta]_{222}/[\Theta]_{208}$ ratio was somewhat lower than that for α-helical content itself (pH 9). The $[\Theta]_{222}/[\Theta]_{208}$ ratio is sensitive to whether the α-helix is single-stranded or in the form of a coiled coil. The winding of the α-helices around each other, or the pitch of the coiled coil, is dependent on the number of amino acids per turn of the α-helix (Phillips, 1992) and varies widely for different proteins (Seo & Cohen, 1993). The number of residues per turn in a right-handed α-helix has been shown to vary between 3.50 and 3.65 in globular proteins (Cohen & Parry, 1990). Correspondingly, the inclination of the "apolar stripe" formed by the residues in positions a and d on the surface of a single α-helix with heptad substructure could vary substantially, and lie anywhere within the range from 0° to 15°, so that the crossing angle between two helices would be from 0° to 30° (Cohen & Parry, 1990). Previous work has suggested that, in principle, CD can be used to measure this pitch angle of the supercoiling, but there may be practical limitations in doing this (Cooper & Woody, 1990). Qualitatively, the data for the CCSL peptide suggests that helix formation and the coiled coil structure are highly linked but may not be completely interdependent.

Stability of the CCSL Structure to Temperature and Urea Denaturation. CD spectral changes were recorded for the CCSL peptide in PBS, over the temperature range of 5–80 °C. The peptide showed a decrease in α-helical signal upon thermal denaturation reflecting the transition from a native folded state to a more disordered one. The temperature melting profile showed a gradual decrease in α-helical content up to 60 °C and then a rapid decrease above 65 °C (Figure 6). Above 65 °C the peptide precipitated out of solution, and upon cooling to room temperature this precipitate remained. These results suggest that as the coiled coil structure unfolds, exposure of the hydrophobic leucine and valine residues leads to aggregation. Since the peptide is insoluble at high temperature, it is only possible to estimate the T_m (temperature required at which 50% of the peptide is in its unfolded form) to be greater than 65 °C. This high T_m indicates that the α-helical structure of the peptide is remarkably stable. Qualitatively, increasing temperature also causes a decrease in $[\Theta]_{222}/[\Theta]_{208}$ ratio as well as in α-helical content (Figure 6). This is consistent with the view that supercoiling and α-helical content are highly coupled.

Urea denaturation also was used to evaluate the stability of the CCSL. As shown in Figure 7, the intensity of the CD signal for the CCSL peptide decreased as the urea concentration was increased, reflecting the loss of α-helical structure. For simplicity the urea denaturation curve was analyzed by assuming a two-state folding/unfolding transition (Pace, 1986), with an awareness that a more complex unfolding equilibrium may exist (Bracken *et al.*, 1988). The values for the transition region were obtained by extrapolating from the linear portions of the denaturation curve at low and high denaturant concentrations (Pace, 1986). A concentration of 3.0 M urea is required to reduce the content of the folded structure to 50%. The inset of Figure 7 shows the free energy associated with the unfolding of the CCSL peptide as a function of urea concentration. The value of the Gibbs free energy in the absence of denaturant (ΔG_u) was estimated by linear

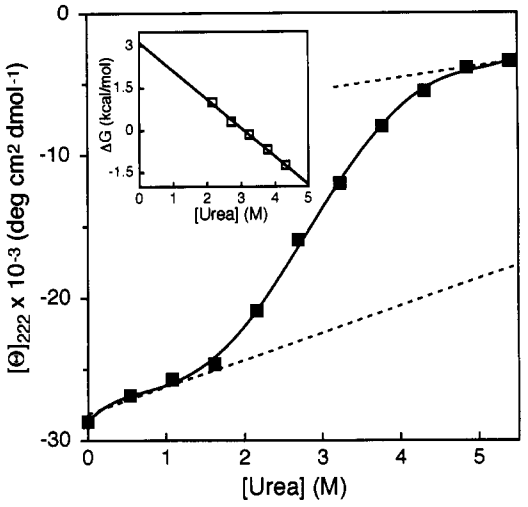

FIGURE 7: Urea denaturation profile of the CCSL peptide. The ellipticity at 222 nm is plotted versus urea concentration. The extrapolated linear pre- and posttransition regions are shown by the dashed lines. Inset: Linear dependence of ΔG_u on the concentration of urea. A free energy of unfolding in the absence of urea of 3.2 kcal/mol was estimated by extrapolating to zero.

extrapolation to zero urea. By this analysis, the folded structure of the CCSL peptide is stabilized, at 25 °C, by a ΔG_u of 3.2 kcal mol^{-1}.

Evidence that the CCSL Peptide forms an Antiparallel Coiled Coil. The CCSL peptide was synthesized with Acm-blocked amino- and carboxy-terminal cysteine residues. These blocking groups were removed and a disulfide bond was formed between the cysteine residues by utilizing oxidation with iodine. As shown in Figure 8, the disulfide form of the CCSL peptide eluted from reverse-phase HPLC much faster that the Acm-blocked peptide. Incubation of the oxidized CCSL peptide in the presence of 5 mM DTT resulted in a shift in the elution position of the reduced peptide back to the time observed for the Acm-blocked peptide (Figure 8, top panel, profile c). Air oxidation of the reduced peptide at pH 7.4 completely converted the peptide back to the disulfide form. In these experiments, Ellman's reagent (Ellman, 1959) confirmed the presence of free thiol groups in the reduced but not the oxidized form of the CCSL peptide.

In PBS, the disulfide form of the CCSL peptide eluted from the size exclusion column as a single peak at the same position observed for the Acm-blocked peptide, suggesting it was a folded monomer. By mass spectral analysis, this peptide gave a molecular mass of 5661 Da (calculated mass = 5645 Da), confirming that the Acm blocking groups had been removed and the peptide had not formed an intermolecular disulfide. However, the observed mass was 16 Da higher than the expected mass of 5645 Da. This increase in the mass was probably due to oxidation of the peptide's single methionine to a sulfoxide as the Acm blocking groups were removed with iodine.

In PBS, the CD spectrum of the disulfide form of the CCSL peptide exhibited a small increase in α-helical content versus the reduced peptide, with no change in the $[\Theta]_{222}/[\Theta]_{208}$ ratio, as shown in Figure 8. These results suggest that the formation of the disulfide bond does not significantly alter the α-helical structure of the CCSL peptide. The oxidized and reduced forms of the peptide showed similar thermal denaturation profiles from 4 to 60 °C, as shown in Figure 8. However, at higher temperatures, the oxidized form showed an increased stability, which was likely the result of the formation of a disulfide bond between the otherwise open ends of the coiled

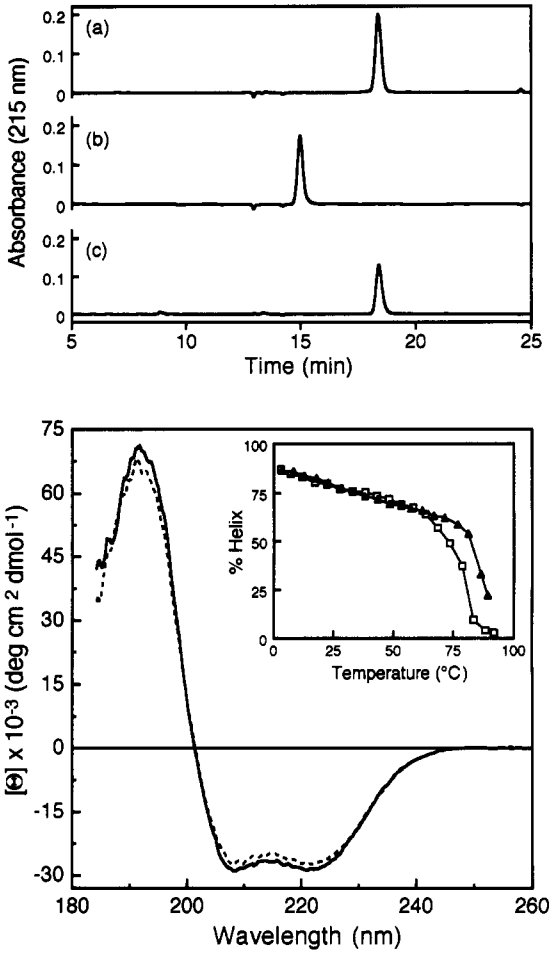

FIGURE 8: Elution profiles and CD spectra of oxidized and reduced forms of the CCSL peptide. (Top) Reverse-phase HPLC elution profiles of the Acm-blocked (a), oxidized (b), and reduced CCSL peptides (c). (Bottom) CD spectra of the oxidized (solid line) and reduced (dashed line) CCSL peptides in PBS, pH 7.4. The reduced sample was run in 1 mM DTT. Inset: Thermal melting profiles for the oxidized (solid triangles) and reduced (open squares) CCSL peptides.

coil. The ability to readily form a disulfide bond between the amino- and carboxy-terminal cysteine residues within the CCSL monomer structure is consistent with the model that the α-helices are stabilized in an antiparallel coiled coil alignment.

CCSL Peptide RGD Binding Activity to GPIIbIIIa. The binding of the CCSL peptide, which contains an RGD sequence in the loop (Figure 1B), to the fibrinogen receptor GPIIbIIIa (Smith *et al.*, 1990) was evaluated and compared with that of the linear control peptide GRGDMP. In competition binding experiments with biotinylated fibrinogen, the GRGDMP and CCSL peptides gave IC_{50} values of 150 and 180 nM, respectively. The ability of the CCSL peptide to compete for binding to the GPIIbIIIa receptor suggests that the RGD sequence present in the loop is available for binding. The slight decrease in affinity for the CCSL peptide versus the linear control peptide GRGDMP may be due to the restricted conformation of the RGD sequence dictated by the CCSL peptide stem loop structure.

DISCUSSION

The goal of the current work was to design a conformationally stable template for the presentation of α-helical and loop peptide sequences in order to construct novel recognition

molecules. Several lines of evidence suggest that the designed CCSL peptide assumes a stable monomeric coiled coil conformation in aqueous solution. (1) It is highly soluble in PBS, which is the first indication that it can assume a folded, globular protein-like conformation consistent with the expected burial of the hydrophobic leucine and valine residues in the coiled coil interface and exposure of the hydrophilic surfaces of the helices. (2) It exhibits an α-helical content approaching 80% in aqueous buffered solutions which is unchanged upon addition of trifluoroethanol (Figure 2), indicating that the peptide is near its maximum helical potential. (3) It has a $[\Theta]_{222}/[\Theta]_{208}$ ratio of 1.02 in aqueous buffer (Figure 2) which is evidence that the α-helices are folded into a coiled coil conformation. (4) Its α-helical content is independent of peptide concentration (Figure 3), suggesting that the α-helices are stabilized through the formation of an intramolecular coiled coil. (5) Size exclusion chromatography and sedimentation equilibrium studies confirm a monomeric molecular mass in aqueous buffer (Figure 4). (6) The peptide exhibits high α-helical content over a wide pH range (Figure 5). (7) The temperature and urea denaturation results indicate that it has a stable folded conformation (Figures 6 and 7). (8) That a disulfide bond readily forms in the monomer between the amino- and carboxy-terminal cysteine residues argues that the α-helices are in an antiparallel alignment. (9) And, the ability of the peptide to compete with fibrinogen for the GPIIbIIIa receptor provides evidence that it assumes a folded structure in which the residues within the loop sequence are both accessible for binding and perhaps conformationally restricted.

The success in achieving the desired conformation of the CCSL peptide makes it useful to examine in detail the design features involved in the prototype structure. The general design of the CCSL peptide was based on the hydrophobic and hydrophilic heptad repeat sequence for noncovalent coiled coils. Hydrophobic interactions at the helical interface of coiled coil proteins appear to provide the major energetic contribution to folding and stabilization of the tertiary or quaternary structure. Parallel and antiparallel coiled coils are distinguishable by particular sequence patterns of these hydrophobic residues that reflect different pairwise interactions across the interface (O'Shea *et al.*, 1991). In the case of a parallel coiled coil as shown in Figure 9A, hydrophobic residues at the heptad positions a and a′ are paired across the interface, as are residues at the d and d′ positions. In contrast, in antiparallel coiled coils as shown in Figure 9B, residues at the heptad positions a and d′ as well as the d and a′ positions are paired across the coiled coil interface (Cusack *et al.*, 1990). In the CCSL peptide, leucine residues were incorporated in the d and a′ heptad positions, as shown in Figure 1, to promote the formation of an antiparallel coiled coil arrangement. Leucine was chosen because of its contribution to the stability of the coiled coil due to its high hydropathy (Zhu *et al.*, 1993). Valine residues were incorporated in the other interface positions a and d′ for a number of reasons. First, valine occurs widely in this same position in "leucine zipper" proteins (Landschulz *et al.*, 1988). Second, at longer chain lengths with only leucine residues occupying all of the interface positions, very stable coiled coils form in a staggered conformation, leading to concatemers because of the "sticky ends" (Talbot & Hodges, 1982). Third, valine residues are thought to stabilize the coiled coil due to packing effects in the hydrophobic core of coiled coils (Zhu *et al.*, 1993).

The CCSL peptide was designed with glutamic acid residues in all of the heptad positions e and g of the amino-terminal

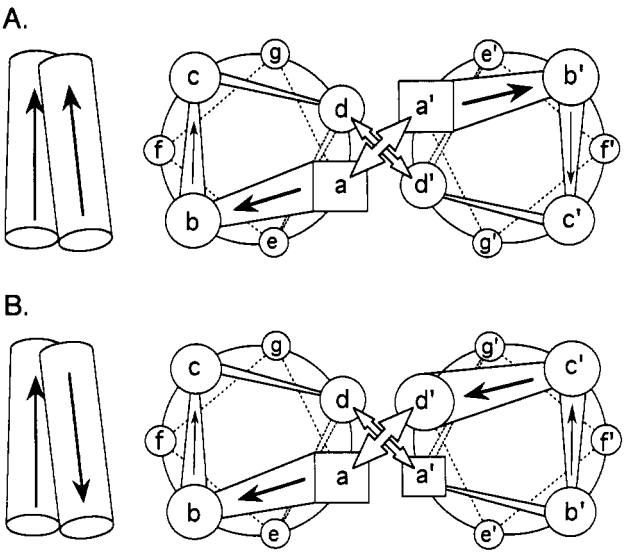

FIGURE 9: (A) Alignment of a parallel coiled coil (left) and the corresponding helical wheel cross section (right) with the heptad positions labeled a, b, c, d, e, f, g, and a′, b′, c′, d′, e′, f′, g′. (B) Alignment of an antiparallel coiled coil (left) and the corresponding helical wheel cross section (right).

α-helix and lysine residues in all of the e′ and g′ positions of the carboxy-terminal α-helix (Figure 1). While salt bridges may make only a small contribution to the total stabilization free energy of the folded coiled coil structure, ion pairs in the border regions of the coiled coil interface are thought to be important in dictating parallel or antiparallel packing (Skolnick & Holtzer, 1985; Mo *et al.*, 1990; O'Shea *et al.*, 1991). In coiled coil sequences residues which border the hydrophobic interface (heptad positions e and g) are often found as complementary ion pairs (Cusack *et al.*, 1990; O'Shea *et al.*, 1991; Zhou *et al.*, 1992a,b). In a parallel coiled coil, the heptad positions e and g′ are paired on one side of the coiled coil interface and positions g and e′ occur together on the other side (Figure 8A). In contrast, in an antiparallel coiled coil, residues at positions e and e′ are paired on one face of the dimer and residues at positions g and g′ occur on the other face (O'Shea *et al.*, 1991) (Figure 8B). The arrangement of charge distribution in the border regions of two separate α-helical peptides, as incorporated here in the CCSL peptide, has been used previously in the successful design of a parallel heterodimeric coiled coil peptide (Graddis *et al.*, 1993). Placing opposite charges at the border positions of the amino- and carboxy-terminal α-helices of the CCSL peptide favors the formation of an antiparallel coiled coil while discouraging the formation of an elongated parallel homodimer because of unfavorable ionic interactions.

In the CCSL peptide, serine and alanine residues occupied the solvent-exposed positions on both α-helical surfaces (heptad positions b, c, and f; b′, c′, and f′) as shown in Figure 1. These residues were not directly involved in stabilizing the coiled coil at the interface. However, they were important to the overall α-helical potential of the individual helices. Alanines were incorporated because of their high helical propensity (Komoriya & Chaiken, 1980; O'Neil & DeGrado, 1990) and ease in chemical synthesis. Serine residues were placed in the solvent-exposed positions because the hydroxyl side chain should increase the solubility of the peptide. The choice of serine was a trade-off because of its lower helical propensity (O'Neil & DeGrado, 1990). Other intrahelix interactions of the type $i \rightarrow i+3$ and $i \rightarrow i+4$ are possible between ionic residues in an α-helix and could add additional stability to the structure

(Sundaralingam *et al.*, 1985; Marqusee & Baldwin, 1987). However, to avoid unpredictable interactions, these types of salt interactions were not incorporated into the CCSL peptide design.

A six-residue loop sequence was incorporated into the CCSL peptide to connect the amino- and carboxy-terminal α-helical segments creating an intramolecular coiled coil. The loop sequence consisted of amino acid residues GRGDMP in positions 26–31, respectively (Figure 1). To prevent the continuous helical rod conformation, the favored α-helical terminator and initiator residues glycine and proline were placed at the beginning and end of the loop sequence, respectively (Presta & Rose, 1988; Richardson & Richardson; 1988). An RGD sequence was incorporated into the loop to serve as a recognition site for GPIIbIIIa binding (Smith *et al.*, 1990). And methionine was incorporated into the loop to serve as a chemical cleavage site (Spande *et al.*, 1970) to test the stability of an intramolecular versus an intermolecular coiled coil structure.

The CCSL peptide contained cysteine residues at the amino- and carboxy-terminal positions corresponding to heptad positions a and d′ across from one another on the α-helical segments (Figure 1). The formation of a disulfide bond between residues at these positions has been used previously to bring two α-helices in register and further stabilize the overall coiled coil structure in particular in the flexible end regions (Hodges *et al.*, 1990; Zhou *et al.*, 1993). However, to avoid the possibility of mixed disulfide formation during peptide purification, the cysteine residues were blocked with acetamidomethyl (Acm). Acm was used because it is thermally stable, easy to remove, and relatively conformationally benign (Holtzer *et al.*, 1990).

Previous work has shown that the interaction of the charged terminal residues with the α-helix dipole can destabilize the α-helix (Fairman *et al.*, 1989). To avoid this helix-destabilizing effect, the α-NH_3^+ and α-COO^- groups of the CCSL peptide were blocked with acetyl and amide groups, respectively (Figure 1) (Marqusee & Baldwin, 1987; Venkatachalapathi *et al.*, 1993).

The minimum chain length for the formation of an α-helical coiled coil is sequence dependent (Talbot & Hodges, 1982). However, a variety of parallel coiled coil peptides with similar design features to the CCSL peptide have been created which suggest that the minimum length for a stable parallel coiled coil peptide is around 28 residues, or 4 heptad repeats (Hodges *et al.*, 1990). The CCSL peptide was designed with 25 residues per α-helix, or three and one-half heptad repeats, for a number of reasons. First, a stability advantage was likely to be gained by joining the coiled coil intramolecularly with a peptide loop sequence similar to the stability gained when intermolecular coiled coils have been linked by a disulfide (Hodges *et al.*, 1990). Second, longer α-helices had the potential of forming stable structures which contain staggered or non-overlapping portions and hence yield multiple coiled coil conformations and aggregation. At shorter chain lengths any staggering of the chains would reduce the hydrophobic interactions to an unacceptable level, and as a result the in-register configuration would be energetically favored (Talbot & Hodges, 1982). Third, longer α-helices would have made the chemical synthesis of the intact CCSL peptide more difficult.

The antiparallel alignment of the α-helices in the CCSL peptide is a unique orientation for *de novo* designed coiled coils, which have been predominately parallel. The antiparallel orientation was achieved by designing specific hydrophobic and ionic interactions within the coiled coil to stabilize the desired conformation while destabilizing undesirable conformations. Competing undesirable conformations may include extended parallel or antiparallel dimers, four-helix bundles, and higher order aggregates. The CCSL peptide is also unique in that it is a single polypeptide designed to spontaneously fold on itself. This design has the major advantage that these peptides will assume the folded conformation independent of concentration and may be expressed *in vivo* or engineered into phage display systems.

The results with the prototype CCSL design indicate that this peptide has a highly stable structure which should permit the replacement of selective amino acids in the noninterface positions and retain the desired conformation. Therefore, this design represents a potential sequence-simplified scaffold into which binding residues from surfaces of α-helices and loop sequences of native proteins and peptides can be inserted to form conformationally constrained mimetic recognition molecules. Most native α-helical sequences consist of helix-permissive or at least helix-passive residues. Such sequences can be directly incorporated into the CCSL template for presentation without disrupting the coiled coil structure. Further, since the α-helix is a regular secondary structure, the spatial arrangement of residues in both native and suitable α-helical mimetics can be predicted. Finally, with the CCSL template it is possible to design a mimetic of either a single α-helix or of two separate helices on the same molecule. Interestingly, the CCSL design is ideally suited for mimicking residues which are presented on an antiparallel alignment of α-helices in such proteins as interleukins 2 and 4 (Bazan, 1992; McKay, 1992; Redfield *et al.*, 1991). In these proteins, residues in helices A and D, which are thought to be involved in receptor interaction, are distant in primary sequence but are folded into an antiparallel coiled coil conformation with respect to one another in the native structure.

Helical and loop mimetic molecules based on the CCSL design may serve several functions. (1) They may represent simplified models for studying macromolecular recognition. (2) They may act as agonists or antagonists of protein–protein or protein–DNA interactions. (3) Positive antagonistic candidates may provide leads in the design of small molecule peptidomimetic drugs. (4) They may be useful to generate neutralizing antibodies directed at conformationally restricted peptide sequences. (5) They may be useful to present α-helical surfaces from antigenic proteins to the immune system as vaccines. (6) The CCSL peptide itself represents an ideal molecule for protein folding studies since it assumes a stable tertiary structure which conformationally mimics elements of native globular proteins.

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