Controlled Formation of Model Homo- and Heterodimer Coiled Coil Polypeptides

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ABSTRACT: Sequence-simplified coiled coil polypeptides were synthesized and their folding properties characterized in order to define the role of charged border residues at the coiled coil interface for the controlled formation of homodimer and heterodimer structures. Three peptides were designed to form parallel coiled coils with valine and leucine occupying the hydrophobic interface positions *a* and *d*, respectively, of the heptad repeat *abcdefg*. The polypeptide designated E/K42, with the heptad repeat sequence VSSLESK, contained glutamate and lysine in the interface border positions *e* and *g*, respectively, and was designed to form a coiled coil homodimer at neutral pH. Two other polypeptides, designated E/E35 and K/K35, have the heptad repeats VSSLESE and VSSLKSK, respectively. E/E35 contains only glutamic acid at both *e* and *g* positions; K/K35, only lysine. E/E35 and K/K35 were designed to form a stable coiled coil heterodimer when combined at neutral pH. All three polypeptides were prepared by solid-phase synthesis and purified by reverse-phase high-performance liquid chromatography followed by size-exclusion chromatography. E/K42 formed a stable dimeric coiled coil structure as determined by circular dichroism and size-exclusion chromatography. The α-helical content of E/K42 was highest at neutral pH and decreased at extremes of pH. The α-helical structure of E/K42 at micromolar concentrations had a T_m of 62–65 °C and exhibited a concentration dependence of thermal denaturation consistent with dimer formation. In contrast to results with E/K42, a mixture of E/E35 and K/K35, but neither alone, forms α-helix at neutral pH. At micromolar concentrations the E/E35:K/K35 mixture had a T_m of 60–63 °C and eluted as a dimer in gel filtration chromatography, suggesting that the peptides form a stable coiled coil heterodimer. Hence, for two peptides, each with a single type of charged residue at all *e* and *g* positions but oppositely charged with respect to each other, heterodimers can be stabilized and homodimers destabilized by charge attraction and repulsion, respectively. In support of this conclusion, the acidic polypeptide E/E35 forms α-helical structure at low pH, while the basic polypeptide K/K35 forms α-helical structure at high pH. The results argue that positions *e* and *g* of the heptad repeat of coiled coil peptides can be varied to control heterodimer and homodimer formation.

The design of recognition molecules is a dominating goal in biotechnology, in order to obtain competitors, mimics, and other ligands for therapeutics, diagnostics, and separation sciences. One approach to the rational design of recognition molecules is through the creation of chimeras composed of conformational domains of naturally occurring proteins which act as carriers or scaffolds to present recognition sequences (Chaiken *et al.*, 1991). Conformational motifs often are stable and excisable subdomains of native proteins. Using them in designing recognition molecules can circumvent the need for total *de novo* design. An example of a chimeric protein containing a conformational motif borrowed from a native protein and onto which a recognition sequence has been grafted is Fc fusion protein (Byrn *et al.*, 1990).

We have been interested to use the coiled coil ("leucine zipper") α-helical motif as a compactly folded unit in constructing chimeric recognition molecules. The leucine zipper domain has been identified in a number of proteins of diverse function (Cohen & Parry, 1986; Lupas *et al.*, 1991) where it serves as a noncovalant dimerization surface for homodimer (O'Shea *et al.*, 1991) and heterodimer assembly (O'Shea *et al.*, 1989a), as well as in intramolecular folding (Cusack *et al.*, 1990). Leucine zippers consist of a heptad repeat, $(abcdefg)_n$ (Figure 1), in which the *d* position is leucine (hence the name), the *a* position is usually a hydrophobic residue, and the *e* and *g* positions are often charged residues (Cohen & Parry, 1990). The high-resolution crystallographic

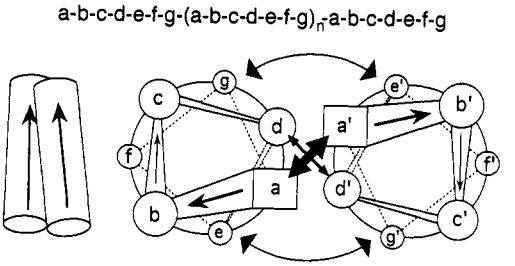

FIGURE 1: Schematic representations of a prototype heptad repeat coiled coil dimer. Top: heptad repeat sequence. Bottom left: parallel coiled coil dimer structure. Bottom right: helical wheel diagram of the coiled coil, which is an end-on view from the amino to the carboxyl ends of parallel, in-register, right-handed α-helices. Arrows indicate the cross-strand interactions between interface postions *a* and *a'* and positions *d* and *d'* and the interactions of the charged border residues in positions *g* and *e'* and positions *e* and *g'*.

structure of the leucine zipper domain of the trans-activating protein GCN4 (O'Shea *et al.*, 1991) shows that residues occupying positions *a* and *d* and the methylene groups of residues at positions *e* and *g* form an interior hydrophobic dimerization surface. In addition, residues at positions *e* and *g* form a border of stabilizing intermolecular salt bridges, while residues at positions *b*, *c*, and *f* are generally exterior and exposed to the solvent.

In the current study, we designed and synthesized several sequence-simplified polypeptides in order to assess the role of the coiled coil interface border residues in the formation of noncovalent homo- or heterodimers, ultimately with controlled

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affinity. The model coiled coil polypeptides described in this report are based on the 4/3 heptad repeat, *abcdefg*, where positions *a* and *d* are valine and leucine, respectively. Residues at positions *e* and *g* are either glutamic acid or lysine, and residues in positions *b*, *c*, and *f* are serine. In particular, a coiled coil peptide, designated E/K42,[1] which contains lysine and glutamic acid in the *e* and *g* positions, respectively, was found to self-associate to form a homodimer. In contrast, a heterodimer coiled coil was formed by two model polypeptides, designated E/E35 and K/K35, which respectively contain only lysine or glutamic acid in the border positions. This work has led to a method for using border residue charges to control homo- and heterodimer formation. A preliminary account of this work was presented at the 12th American Peptide Symposium, Cambridge, MA, in June, 1991 (Graddis & Chaiken, 1992).

EXPERIMENTAL PROCEDURES

Materials. Solid-phase peptide synthesis resins were obtained from Applied Biosystems, Inc. (Foster City, CA). Protected amino acids were purchased from Bachem (Philadelphia, PA), Keystone Bio-Tec (Philadelphia, PA), and Peninsula Laboratories (Belmont, CA). Diisopropylcarbodiimide was obtained from Milligen/Biosearch (Burlington, MA). Diisopropylethylamine, hydroxybenzotriazole, 1-methyl-2-pyrrolidinone, anisole, dimethyl sulfide, *m*-cresol, ethanedithiol, piperidine, pyridine, and buffer salts were obtained from Aldrich (Milwaukee, WI). All solvents were HPLC grade and were purchased from Baker (Philipsburg, NJ) or Mallinckrodt, Inc. (Paris, KY). Dialysis membrane, MW 1000 cutoff, was obtained from Spectrum (Los Angeles, CA). HPLC columns were from Vydac (Hesperia, CA) and Beckman (Palo Alto, CA).

Peptide Synthesis. Peptides were assembled using solid-phase methodology on a Biosearch 9500 synthesizer, employing the *tert*-butylcarbonyl (*t*-BOC) strategy. Side-chain protecting groups used were Asp (hexyl ester), Glu (benzyl ester), Cys (*p*-methoxybenzyl), Lys (2-chlorobenzyloxycarbonyl for E/E35 and K/K35; trifluoroacetyl for E/K42), Ser (benzyl), and Tyr (2-bromobenzyloxycarbonyl). Peptide E/K42 was synthesized on a *t*-BOC-leucine hydroxymethyl phenylacetamidomethyl resin. The completed peptide was cleaved from the resin by using a modified hydrogen fluoride cleavage method (Tam, 1988), washed with ether, and extracted with TFA. The TFA was evaporated on a rotary evaporator, and the peptide was dissolved in 10% acetic acid, applied to a C8 reverse-phase cartridge, eluted with 80% actetonitrile/1% TFA, and lyophilyzed. The lysine TFA protecting group was removed by treatment with 1.0 M piperidine at 4 °C for 4 h (Goldberger & Anfinsen, 1962), dialyzed against 1.0 M ammonium bicarbonate, pH 8, and lyophilized.

Peptides E/E35 and K/K35 were assembled on *p*-methylbenzhydrylamine resin. Completed peptides were removed from the resin by using the "low-high" TFSMA cleavage method (Tam & Merrifield, 1987). Peptides were extracted with TFA; precipitated in cold ether by addition of up to 1% pyridine; collected by centrifugation; dialyzed against 1.0 M ammonium bicarbonate, pH 8, followed by 10 mM ammonium bicarbonate, pH 8; and lyophilized.

All crude peptides were initially purified by reverse-phase HPLC using a Vydac C4 column eluted with a linear gradient from 10 to 90% acetonitrile containing 0.1% TFA. The peptides were further purified by reverse-phase HPLC using a Beckman C18 column. The E/K45 and K/K35 peptides were eluted with a linear gradient from 0 to 70% acetonitrile containing 10 mM phosphate, pH 3. The E/E35 peptide was eluted with the same gradient, using 10 mM phosphate, pH 7. The final purification step was gel filtration chromatography on a 2.5 × 25 cm Superose 12 column eluted with PBS. Chromatographic eluent was monitored at 226 nm for E/K42 and 280 nm for E/E35 and K/K35.

The purities of the peptides were verified to be greater than 90% by reverse-phase HPLC using a C18 column (Vydac, 4.6 mm × 25 cm). Elution was done with 0.05% TFA and a gradient of 0–80% acetonitrile, 0.5 mL/min, for 60 min. Quantitative amino acid analysis after acid hydrolysis (Beckman 6300 amino acid analyzer) yielded the following compositions, with expected values in parentheses; these are consistent with intended sequences. E/K42: Ser, 17.5 (18); Glu, 6.5 (6); Val, 6.0 (6); Leu, 6.3 (6); Lys, 5.6 (6). E/E35: Asp, 1.0 (1); Ser, 11.6 (13); Glu, 8.6 (8); Gly, 1.8 (2); Val, 4.2 (4); Leu, 4.3 (4); Tyr, 1.0 (1); Lys, 1.1 (1); Cys, not determined (1). K/K35: Asp, 1.0 (1); Ser, 11.8 (13); Gly, 2.0 (2); Val, 4.3 (4); Leu, 4.5 (4); Tyr, 1.0 (1); Lys, 9.3 (9); Cys, not determined (1). Peptide sequencing for E/K42 with an Applied Biosystems 490A sequencer verified the intended sequence through 15 cycles. E/E35 and K/K35 were *N*-acetylated and hence could not be sequenced. Fast atom bombardment mass spectrometry for E/K42, with an M-Scan's VG Analytical ZAB 2-SE high-field mass spectrometer operating at V_{acc} = 8 KV, gave an M_r of 4403.5 Da (vs 4402.9 Da calculated from the sequence). For E/E35 and K/K35, mass spectral analyses (matrix-assisted laser desorption time of flight) revealed molecular masses within 1% of theoretical. However, the presence of multiple alkali metal adducts that broadened the observed peaks made it impossible to accurately assign M_r values. Concentrations of stock peptide solutions used for gel filtration and circular dichroism experiments were determined by amino acid analysis.

Gel Filtration Chromatography. Gel filtration chromatography was performed on a 30/16 Superdex-75 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 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 carbonic anhydrase, cytochrome *c*, and aprotinin (M_r = 29, 12.4, and 6.5 kDa, respectively). Experiments with E/E35 and K/K35 were done in the presence of 1 mM DTT.

Circular Dichroism Measurements. Circular dichroism spectra of peptides were obtained using a Jasco 500 C spectropolarimeter interfaced with an AST AT computer. Raw data (ellipticities) were processed, after averaging and correction for appropriate solvent blanks, according to eq 1:

$$\mathrm{MRE} = [(\theta_{obs})(\mathrm{MRW})]/10lc \quad (1)$$

where MRE is the mean residue ellipticity in (deg)(cm^2)/dmol, θ_{obs} is the observed ellipticity in mdeg, MRW is the

[1] Abbreviations: E/K42, 42-residue leucine zipper polypeptide with Glu and Lys in heptad repeat positions *e* and *g*, respectively; E/E35, 35-residue leucine zipper polypeptide with Glu in heptad repeat positions *e* and *g*; K/K35, 35-residue leucine zipper polypeptide with Lys in heptad repeat positions *e* and *g*; E/E35:K/K35 heterodimer, the noncovalent complex of E/E35 and K/K35; CD, circular dichroism; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; M_r, molecular mass; MRE, mean residue ellipticity; MRW, mean residue molecular mass; PBS, phosphate-buffered saline; *t*-BOC, *tert*-butyloxycarbonyl; TFA, trifluoroacetic acid; TFE, trifluoroethanol.

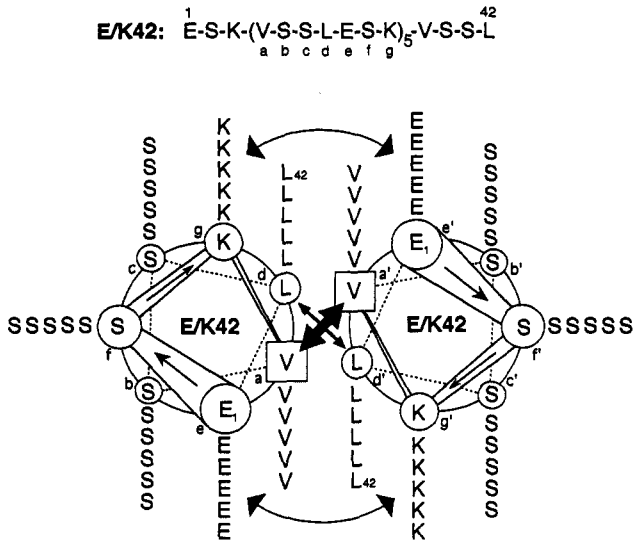

FIGURE 2: Linear sequence and helical wheel diagram of the 42-residue polypeptide E/K42 with the haptad repeat positions labeled a, b, c, d, e, f, and g.

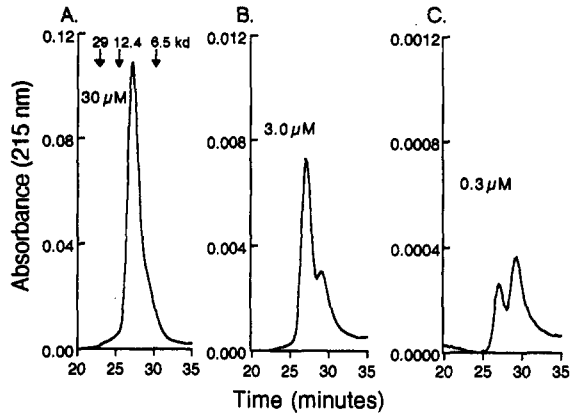

FIGURE 3: Gel filtration chromatography elution profiles for E/K42 loaded at concentrations of 30 (A), 3 (B), and 0.3 μM (C). The Superdex 75 column was run at 22 °C in 150 mM sodium chloride and 20 mM phosphate, pH 7.4, at a flow rate of 0.5 mL/min, and column effluent was monitored at an absorbance of 215 nm. The arrows indicate the positions of the globular protein standards carbonic anhydrase, cytochrome *c* and aprotinin with molecular masses corresponding to 29, 12.4, and 6.5 kDa, respectively.

mean residue weight (105 for E/K42, 104.4 for E/E35, and 104.2 for K/K35), *l* is the cell path length in cm, and *c* is the concentration of peptide in g/mL. All spectra were measured at 270–185 nm using a 1-nm bandwidth and a 1-s time constant. The percent α-helical content was estimated from the ellipticity minimum at 222 nm using the method of Chen *et al.* (1974). For experiments with E/E35 and K/K35, 1 mM DTT was present.

To test the effect of the α-helix inducing solvent TFE on α-helical content, CD spectra were recorded for the peptides in PBS alone and in increasing concentrations of TFE.

To determine the effect of pH on α-helical content, CD spectra of the peptides were measured from pH 2 to 12. Stock peptide solutions were prepared with 150 mM sodium chloride in dilute phosphoric acid, 100 mM phosphate, and dilute sodium hydroxide. The desired pH was generated by mixing the appropriate buffered peptide solutions. The peptide samples were incubated at the desired pH for 1 h before the CD spectra were taken.

Melting curves were obtained by measuring $[\theta]_{222}$ as a function of temperature. The temperature was adjusted with a thermostated Neslab RT-210 circulating water bath equipped with an MTP-6 microprocessor and then checked with a thermocouple inserted directly into the cell. The temperature was raised at a rate of 0.5 °C/min. Four separate melting curves for each peptide concentration were averaged to produce thermal melting profiles, which were corrected for temperature-dependent drift of pre- and posttransition baselines to calculate the fraction of unfolded peptide.

RESULTS

Design of E/K42 Homodimer. Peptide E/K42 was designed to form a homodimer whose quaternary structure consisted of two α-helices arranged in a parallel coiled coil. The E/K combination represents a simplified sequence of known coiled coil proteins, such as tropomyosin (McLachlin & Stewart, 1975) and the well-characterized leucine zipper domains of Jun and Fos (O'Shea *et al.*, 1989b), GCN4 (O'Shea *et al.*, 1989a), and C/EBP (Landschulz *et al.*, 1988). As shown in Figure 2, there are five internal heptad repeats (*abcdefg*) in E/K42. Hydrophobic interactions at the helical interface of coiled coil proteins appear to provide the major energetic contribution to folding and stabilization of the coiled coil structure. In the E/K42 peptide, valine and leucine occupy the *a* and *d* positions, respectively. These residues were chosen due to their high occurrence at these positions in coiled coil proteins and leucine zipper domains of DNA binding proteins (Cohen & Parry 1990). As noted recently (Zhu *et al.*, 1993), leucine residues in the *d* position appear to provide the greatest stabilization of the coiled coil due to hydropathy characteristics, while valine and other β-branched residues in the *a* position provide favorable packing in the coiled coil interface.

In the E/K42 peptide, the coiled coil interface border positions *e* and *g* are occupied by glutamic acid and lysine, respectively. The side-chain methylene groups of these residues extend the hydrophobic interface, while the charged groups form intermolecular salt bridges between the coiled coils. The helical wheel diagram in Figure 2 shows the interchain interactions of the glutamic acid and lysine across the coiled coil interface at positions *e*–*g'* and *g*–*e'*. These interactions favor the formation of a parallel homodimer coiled coil.

The positions not involved in the dimer interface, *b*, *c*, and *f*, are occupied by serine. Serine was chosen primarily to increase the solubility of the peptide while not introducing charged residues that may interfere with the intermolecular salt bridges formed by residues at positions *e* and *g*. The choice of serine in the *b*, *c*, and *f* positions is a tradeoff between its use to form a noncharged hydrophilic surface and its tendency to decrease α-helical propensity (Lyu *et al.*, 1990).

Gel Filtration of E/K42. The E/K42 peptide eluted from the gel filtration column as two peaks (Figure 3). The proportions of the peaks changed as the concentration of the loaded peptide was lowered. The faster eluting peak predominated when the peptide was loaded at a concentration of 30 μM (Figure 3A). However, as the starting peptide concentration was decreased to 0.3 μM (Figure 3C), this peak decreased and the slower eluting peak increased to become the dominant peak. With the column calibrated with standard globular proteins, the elution volume of the faster eluting peak corresponds to an apparent molecular mass of 10 000 Da. This value is close to the calculated dimeric molecular mass for E/K42, which is 8800 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

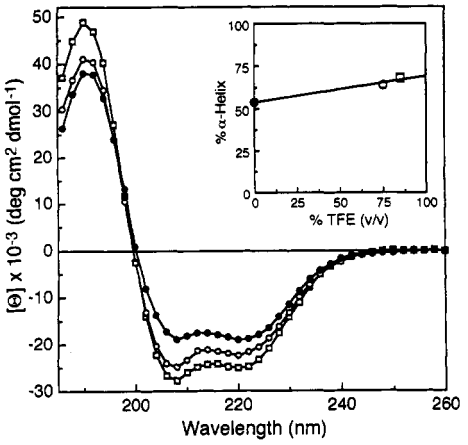

FIGURE 4: Circular dichroism spectra of 5 μM E/K42 in the presence and absence of trifluoroethanol (TFE) recorded in 10 mM sodium phosphate, pH 7.4: 0% TFE (●), 75% (v/v) TFE (○), and 85% (v/v) TFE (□) at 25 °C. Inset: TFE concentration versus percent α-helical content.

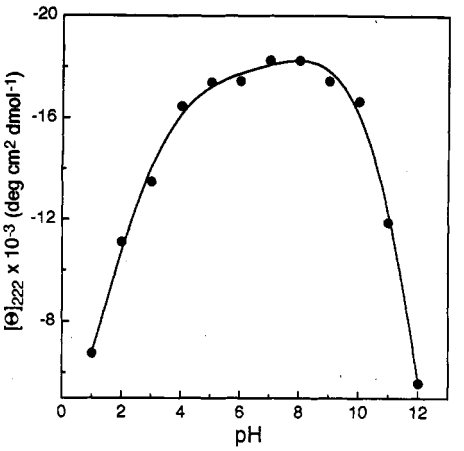

FIGURE 5: pH dependence on mean molar ellipticity at 222 nm for E/K42. The $[\theta]_{222}$ values were determined from CD spectra recorded using 6 μM peptide in 100 mM sodium phosphate, 25 °C, at the pH conditions shown.

concentration is lowered. The slightly larger size determined for the dimer E/K42 peptide by gel filtration is consistent with results seen for other coiled coil peptides. In benign medium, α-helical coiled coils are rodlike in shape and elute more rapidly from a gel filtration column than globular molecules of identical molecular mass (Hodges *et al.*, 1981). The elution position and behavior of E/K42 peptide at different concentrations during gel filtration lead to the conclusion that the peptide forms a dimer in solution.

Circular Dichroism of E/K42. In aqueous buffer, E/K42 exhibits an α-helix-like circular dichroism spectrum with large molar ellipticity minima values at 222 and 208 nm and a positive transition at 192 nm (Figure 4). The close equivalence of the molar ellipticity minima at 222 and 208 nm is similar to that observed before for coiled coils (Cooper & Woody, 1990; Zhou *et al.*, 1992a) and distinctly different from the relatively smaller negative ellipticity at 222 vs 208 nm found for single-stranded α-helices in peptides and proteins (Cooper & Woody, 1990). The ellipticity minimum at 222 nm was used to estimate (Chen *et al.*, 1974) a 55% α-helical content for E/K42 at a concentration of 5 μM at 25 °C. The helical content was not significantly decreased upon reduction of the E/K 42 concentration down to 25 nM, the lower limit of detectability. Upon addition of the helix-inducing solvent trifluoroethanol (TFE) to 75%, helical content increases by only 8% (Figure 4). However, even in the presence of higher TFE concentrations, the overall helical content never reaches 100%.

The addition of TFE causes a blue shift and an increase in the ellipticity minimum around 208 nm for the E/K42 peptide (Figure 4). A decrease and a red shift in ellipticity minima have been shown to correspond to conversion of a rigid single-stranded α-helix to an α-helical coiled coil structure (Cooper & Woody, 1990; Zhou *et al.*, 1992b). Previous studies on parallel coiled coil peptides found a molar ellipticity ratio at 222 and 208 nm ($[\theta]_{222}/[\theta]_{208}$) of about 1.03 for a highly coiled two-stranded coiled coil in aqueous solutions (Lau *et al.*, 1984; Hodges *et al.*, 1988, 1990; Zhou *et al.*, 1992a) and about 0.86 for a single-stranded α-helix in the presence of TFE (Zhou *et al.*, 1992b). For the E/K42 peptide in aqueous buffer at 25 °C the magnitude of the $[\theta]_{222}/[\theta]_{208}$ ratio is 1.02, evidence that the α-helices are stabilized in a coiled coil conformation. In 75% TFE, the $[\theta]_{222}/[\theta]_{208}$ ratio changes to 0.90, indicative of a single-standed α-helix in this condition. These results lead to the conclusion that in aqueous solution the E/K42 peptide assumes a coiled coil structure.

pH and Thermal Denaturation of E/K42. The data presented in Figure 5 show the variation of ellipticity of E/K42 at 222 nm as a function of pH. E/K42 forms a stable α-helical structure from pH 4 to 10. At extremes of pH, the α-helical content decreases. This decrease presumably is caused by the loss of Glu–Lys salt bridges, between the charged residues at the *e* and *g* border positions, that contribute to stabilization of the coiled coils between the pH 4 and 10 positions. Interestingly, previously studied coiled coils with Glu and Lys in border positions *e* and *g* (Lowey, 1965; Lau *et al.*, 1984; Zhou *et al.*, 1992c) have found less abrupt decreases in helical stability at low pH than observed here for E/K42. The reason for this difference is not fully understood at present.

Temperature-induced unfolding of E/K42 was characterized by CD analysis. The data obtained are shown in Figure 6. The isodichroic point at 203 nm is consistent with a two-state helix-to-random transition (Greenfield & Fasman, 1969). Unfolding at elevated temperature was found to be reversible as judged by return to the native-like CD spectrum upon cooling. An example of the temperature dependence of the CD signal at 222 nm is shown in Figure 6A for E/K42 at 10 μM. The T_m for the helix-to-random transition increases with increasing peptide concentration as shown in Figure 6B. At micromolar concentrations, E/K42 has a T_m in the range 62–65 °C. The relatively high T_m and its concentration dependence indicate that the α-helical structure is due to a multimeric, not a monomeric, species.

Design of E/E35 and K/K35 Heterodimer. Peptides E/E35 and K/K35 were designed to form a coiled coil heterodimer with the linear sequences shown in Figure 7. For both peptides, positions *a* and *d* are occupied by valine and leucine, respectively, to form the hydrophobic interior of the dimer interface. This hydrophobic interaction across the dimer interface is shown in the helical wheel diagrams in Figures 1 and 7 and is the same hydrophobic core as in the E/K42 homodimer peptide.

The coiled coil interface border positions *e* and *g* are occupied by glutamic acid in the E/E35 peptide and by lysine in the K/K35 peptide. Intermolecular salt bridges formed between these residues, as shown in Figure 7, were predicted to promote the formation of a stable heterodimer between these two peptides, while the formation of homodimers between either of these peptides should be disfavored at neutral pH due to repelling charges.

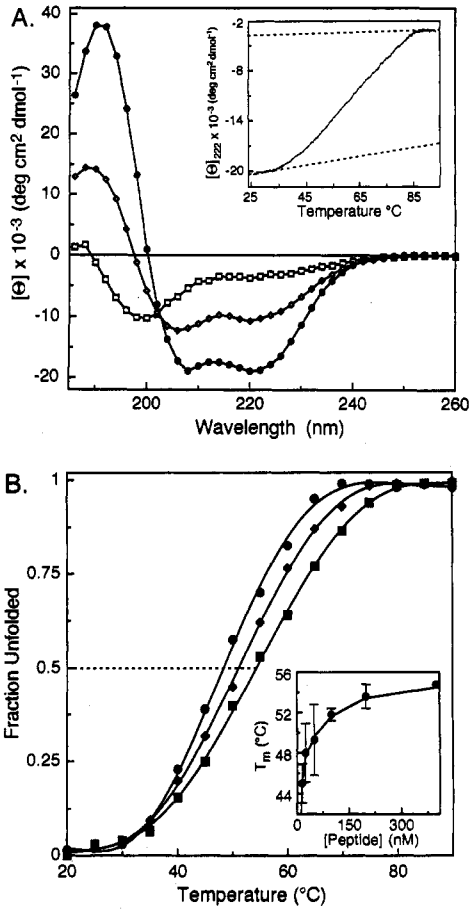

FIGURE 6: (A) Circular dichroism spectra of E/K42 as a function of temperature. The CD spectra of 10 μM E/K42 were recorded in 10 mM sodium phosphate, pH 7.4, at 25 °C (●), 65 °C (◇) and 90 °C (□). Inset: Thermal melting profile of 10 μM E/K42, $[\Theta]_{222}$ versus temperature. The dashed lines represent the pre- and posttransition phases. (B) Concentration dependence of the thermal denaturation of E/K42. The fraction of unfolded peptide was determined by monitoring the CD spectra at 222 nm of E/K42 polypeptide in 10 mM sodium phosphate, pH 7.4: 25 (○), 100 (◇), and 200 nM (□). Data sets are the average of three independent determinations. Inset: The temperature at which the polypeptide E/K42 is 50% unfolded, T_m, as a function of peptide concentration.

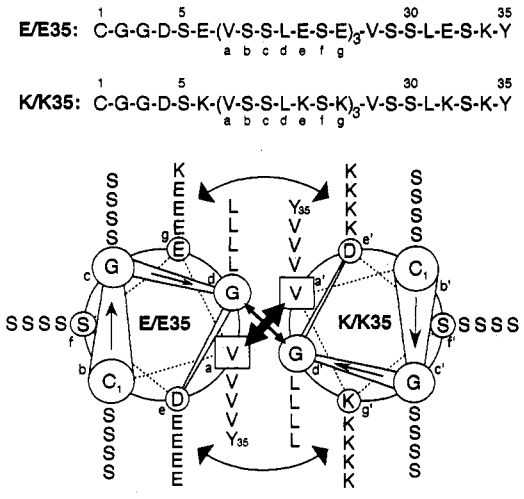

FIGURE 7: Linear sequences and helical wheel diagrams of the 35-residue polypeptides E/E35 and K/K35, with the haptad repeat positions labeled a, b, c, d, e, f, and g and a′, b′, c′, d′, e′, f′, and g′, respectively.

The positions in the heptad repeats not involved in the dimer interface for the heterodimer peptides E/E35 and K/K35

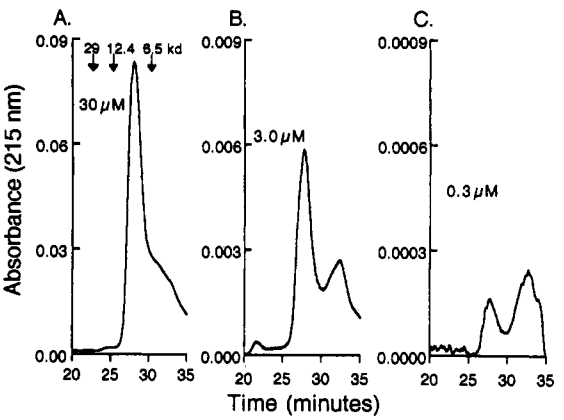

FIGURE 8: Gel filtration chromatography elution profiles for the 1:1 mixture of E/E35 and K/K35 loaded at concentrations of 30 (A), 3 (B), and 0.3 μM (C), respectively. The Superdex 75 column was run at 22 °C in 150 mM sodium chloride, 20 mM phosphate, pH 7.4, and 1 mM DTT at a flow rate of 0.5 mL/min, and column effluent was monitored at an absorbance of 215 nm. The arrows indicate the positions of the globular protein standards carbonic anhydrase, cytochrome *c*, and aprotinin with molecular masses corresponding to 29, 12.4, and 6.5 kDa, respectively.

(positions *b*, *c*, and *f*), as with the homodimer peptide E/K42, are occupied by serine. At the amino terminus of each heterodimer peptide, a cysteine was incorporated to stabilize the potential coiled coil structure by disulfide formation. As it turned out, the heterodimer was sufficiently stable as a noncovalent dimer to be able to avoid the added complexity of a covalent disulfide attachment. An aspartic acid and a lysine were placed at the amino and carboxyl termini, respectively, of the sequences in order to stabilize the α-helix by helping to neutralize the helix macrodipole (Shoemaker *et al.*, 1987). The carboxyl-terminal tyrosine was included to provide absorbance at 280 nm and help determine peptide concentration.

Gel Filtration of the Mixed E/E35 and K/K35 Peptides. A 1:1 mixture of the E/E35 and K/K35 peptides eluted from the gel filtration column as two peaks, and the proportions of the peaks changed as the concentration of the loaded peptide was lowered (Figure 8). The faster eluting peak predominated at high peptide concentrations and appeared to convert into the slower eluting peak at lower concentrations (Figure 8). The elution volume of the faster eluting peak corresponds to an apparent molecular mass of 9000 Da. This value is close to the calculated dimeric molecular mass for the E/E35:K/K35 heterodimer, which is approximately 7400 Da. The slower eluting peak corresponds in elution volume to the individual polypeptides E/E35 and K/K35 when these are run separately and hence represents the monomeric forms of the peptides which are in equilibrium with the dimeric state.

Circular Dichroism of E/E35:K/K35 Heterodimer and Component Peptides. The CD spectra of E/E35, K/K35, a 1:1 molar mixture of the two peptides at neutral pH, and a 1:1 mixture in 50% TFE are shown in Figure 9. E/E35 and K/K35 by themselves exhibit CD spectra typical of random structure, with a maximum negative transition centered at 202 nm. In contrast, an equimolar mixture of the two peptides displays significant α-helical content as indicated by the negative minima at 222 and 208 nm and a positive transition at 192 nm. The ellipticity minimum at 222 nm was used to estimate a 38% α-helical content for the E/E35:K/K35 complex in benign solutions, assuming 35 residues per polypeptide (Chen *et al.*, 1974). The α-helical content of the E/E35:K/K35 mixture is increased by only 3% upon addition of 50% TFE, as judged by the 222-nm minimum. Higher

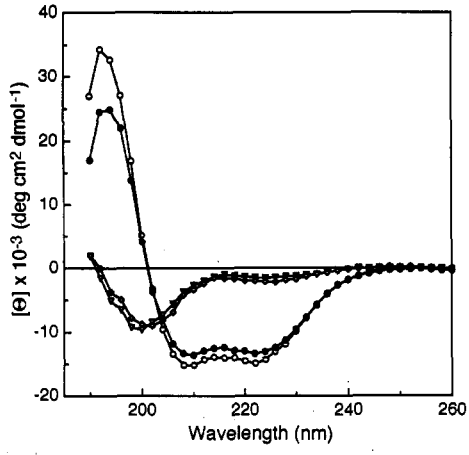

FIGURE 9: Circular dichroism spectra of E/E35, K/K35, and an equimolar mixture of these peptides. CD spectra of 100 μM E/E35 (▽), 100 μM K/K35 (◇), 50 μM E/E35 and 50 μM K/K35 (●), and 50 μM E/E35 and 50 μM K/K35 in 50% TFE (○) were recorded in 10 mM sodium phosphate and 1 mM DTT, pH 7.4, at 25 °C.

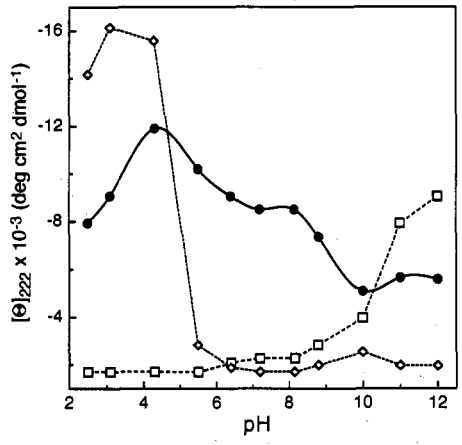

FIGURE 10: pH dependence of α-helicity of E/E35, K/K35, and an equimolar mixture of these peptides, measured by variation of molar ellipticity at 222 nm. The $[\theta]_{222}$ values were determined from CD spectra recorded from 5 μM E/E35 (◇), 5 μM K/K35 (□), and a mixture of 2.5 μM of each (●), in all cases at the pH conditions shown, with 1 mM DTT at 25 °C.

TFE concentrations failed to produce a significant further increase in α-helical content of E/E35:K/K35.

pH and Thermal Denaturation of the E/E35:K/K35 Heterodimer. The data presented in Figure 10 show the MREs of E/E35, K/K35, and the E/E35:K/K35 mixture at 222 nm as a function of pH. Both peptides alone exhibit random coil-like spectra around neutral pH. This is presumably due to an inability to form stabilizing salt bridges between border residues *e* and *g*, since these are all Glu in E/E35 and all Lys in K/K35. In contrast, the solution of E/E35 begins to show significant α-helical content below pH 5, where removal of charged groups upon protonation of carboxylates presumably permits some stabilization of E/E35 homodimer coiled coil. Analogously, K/K35 begins to show α-helical content above pH 9, where removal of positive charges by Lys deprotonation permits formation of stable K/K35 homodimer. In both cases, the increased coiled coil homodimer formed upon neutralization of charged groups in positions *e* and *g* likely results from stabilization from hydrophobic interactions of border residue pairs (Glu–Glu and Lys–Lys, respectively) when charge repulsion effects are removed. The greater negative ellipticitiy of E/E35 at low pH than K/K35 at high pH may reflect greater hydrophobic interactions of protonated Glu (in E/E35 at low pH) than deprotonated Lys (in K/K35 at high pH).

In contrast to the E/E35 and K/K35 peptides alone, the E/E35:K/K35 mixture shows significant α-helical content around neutrality, between pH 6 and 8, consistent with formation of heterodimer under these conditions. At extremes of pH, the mixture exhibits an α-helical content consistent with homodimer formation of one or the other of the component peptides. The ellipticities are lower for the E/E35:K/K35 mixture than for either peptide component alone because the concentration of associating peptides in the mixture is one-half (5 μM) that in samples of the separate species (10 μM). These data show that E/E35 and K/K35 form coiled coil homodimers at acidic and basic pH, respectively, while the E/E35:K/K35 mixture is capable of forming a coiled coil heterodimer at neutral pH.

The stability of the E/E35:K/K35 heterodimer was assessed by measuring the thermal denaturation of helicity using circular dichroism. Thermal melting profiles obtained (data not shown) were very similar to those for E/K42 (Figure 6A). The T_m values of E/E35:K/K35 in the 1–40 μM concentration range were in the range 60–63 °C. The relatively high stability of the E/E35:K/K35 mixture indicates that the α-helical content is stabilized through the formation of multimeric species, consistent with heterodimer.

DISCUSSION

The work presented here demonstrates how charged residues can be used to control the formation of stable homo- and heterodimer coiled coils. The simplified sequences made to demonstrate these design features all contain three main elements: (i) hydrophobic residues at positions *a* and *d* to stabilize helix dimerization through hydrophobic and van der Waals interactions; (ii) charged residues at positions *e* and *g* in order to form interhelical electrostatic interactions; and (iii) hydrophilic residues at positions *b*, *c*, and *f* to form exposed helical surfaces amenable to the aqueous environment. The homo- and heterodimer peptides contain similar sequence elements i and iii. What distinquishes them is the nature of the charged residues at *e* and *g* (sequence element ii). Coiled coil homodimer E/K42 was constructed with glutamic acid and lysine in the *e* and *g* positions, respectively. This combination of charges produces Glu–Lys salt bridges to stabilize the homodimer. In contrast, the heterodimer formed by E/E35 and K/K35 is composed of monomers having either glutamic acid or lysine at both *e* and *g* positions. This sequence combination forms stabilizing Glu–Lys salt bridges as heterodimer, while homodimers are destabilized by potential Glu–Glu and Lys–Lys repulsions at neutral pH.

Several lines of evidence suggest that E/K42 forms a stable dimeric quaternary structure. First, the elution behavior of the purified peptide in size-exclusion chromatography shows a molecular mass of 10 000 Da. This value is close to the expected value of approximately 8770 Da for a dimer of the E/K42 sequence. Second, the greater negative ellipticity at 222 vs 208 nm ($[\theta]_{222}/[\theta]_{208} = 1.02$) is similar to the ratio of 1.03 observed with other coiled coil systems (Cooper & Woody, 1990). Third, the relatively high T_m for thermal denaturation of the α-helical structure of E/K42 (62 °C at a concentration of 10 μM) is comparable to T_m's obtained for other native and *de novo* leucine zippers (Lau *et al.*, 1984; O'Shea *et al.*, 1989). Such stability is not expected for single-stranded helices. Fourth, the concentration dependence of thermal denaturation of E/K42 is indicative of oligomerization and is observed with noncovalent coiled coils (O'Shea *et al.*, 1989; De Francesco *et al.*, 1991).

Polypeptides E/E35 and K/K35 were designed to explore the requirements for heterodimer formation. These two polypeptides contain respectively the 4/3 heptad repeats VSSLESE and VSSLKSK. These sequences differ from homodimer E/K42 in being singly charged in both *e* and *g* positions. One, E/E35, is negatively charged, while the other, K/K35, is positively charged. The data in Figures 8–10 show that these polypeptides form heterodimer at neutral pH. In particular, the gel filtration data in Figure 8 show a molecular mass for the E/E35:K/K35 complex of 9000 Da, close to the calculated value of 7400 Da expected for heterodimer. Neither of the component peptides of the heterodimer exhibits greater than monomer molecular mass by gel filtration. In addition, the CD spectra in Figures 9 and 10 show coiled coil formation of E/E35:K/K35 heterodimer but not of either component alone. The high T_m of the E/E35:K/K35 complex suggests a stability similar to that for E/K42. Taken together, the data argue that the E/E35:K/K35 mixture forms a stable, pH-dependent heterodimer. This result extends the recent observation that the *e* and *g* positions modulate heterodimer formation in the natural-sequence Jun–Fos oncoprotein heterodimer (O'Shea *et al.*, 1992).

While gel filtration, circular dichroism, and stability properties indicate that both E/K42 and the mixture E/E35:K/K35 form dimer coiled coils, the absolute values of ellipticities at 222 nm reflect less than 100% helix. Ellipticity minima observed with increasing peptide concentrations suggest helix contents of 60% and 40%, respectively, for E/K42 and E/E35:K/K35. Helical content is increased, but only incrementally, by TFE. Even at high concentrations, TFE does not induce greater than 75% helix in E/K42 and 45% in E/E35:K/K35. While TFE induces helicity in a potentially α-helical polypeptide (Nelson & Kallenbach, 1986), it appears not to do this indiscriminately (Hodges *et al.*, 1990). That is, regions that have a strong tendency to adopt an α-helical conformation become more α-helical in TFE, but regions that are helix-disfavoring are not induced to become α-helical (Nelson & Kallenbach, 1986, 1989; Hodges *et al.*, 1990). The inability of TFE to increase the helical content of E/K42 and E/E35:K/K35 to 100%, even at very high TFE concentrations, indicates that under these conditions (pH 7.4 and 25 °C) the peptides are near their maximum α-helical formation.

In the current study, all peptides contain a large number of Ser residues, placed in positions *b*, *c*, and *f*. These were included to create a noncharged hydrophilic exterior (Figures 1 and 7). Serine has only a modest α-helical propensity (O'Neil & Degrado, 1990), and adjacent serines in positions *b* and *c* may exert a cooperative destabilizing effect on helix structure (Lyu *et al.*, 1990). In addition, the ends of the heterodimer sequences in both cases contain residue sequences that are nonheptad repeat and hence would not be expected to form stable helical structure. This is particularly evident for E/E35 and K/K35, where only 25 residues conform to the heptad repeat sequence. All of these factors could contribute to the less than 100% helical content of the model peptides. However, it needs to be emphasized that while helical content of the simplified sequence homo- and heterodimer coiled coils is significantly less than 100%, the stabilities are nonetheless quite high.

Importantly, the less than 100% helix found for simplified sequence coiled coil dimers in the present study is consistent with the range of maximum helicity reported previously for synthetic coiled coils. O'Shea et al. (1989b) observed a helix content of 70% for a 43-residue Fos leucine zipper. And, De Francesco et al. (1991) observed a maximum helicity of 60% for a 32-residue polypeptide that mimics the proposed coiled coil dimerization domain of transcription factor LFB1. Interestingly, near 100% helicity has been observed for some designed coiled coil proteins (Engel *et al.*, 1991; Hodges *et al.*, 1990), so that perfecting sequence to increase helix content certainly is possible for such sequences as the homo- and heterodimers made in the current study.

Several investigators recently have reported the characterization of other model leucine zipper homodimer polypeptides. The 4/3 heptad repeats (*abcdefg*) that are used in these studies to form stable coiled coils include IEAIEAR (Engel *et al.*, 1991), LEALEGK (Hodges *et al.*, 1990), and LEALEKK (O'Neil *et al.*, 1990). These and additional studies that compare the amino acid sequence of heptad repeats from numerous native leucine zippers (Lupus *et al.*, 1991; O'Shea *et al.*, 1993) and coiled coil fibrous proteins (Lau *et al.*, 1984) indicate that the coiled coil motif is highly tolerant of amino acid substitutions as long as these conform to the general hydrophilic–hydrophobic profile of the 4/3 heptad repeat found in coiled coil proteins. This tolerance for amino acid substitutions is reflected in the successful coiled coil sequence simplifications accomplished in the current study.

Overall, the homodimer and heterodimer coiled coils reported in this paper may be useful as scaffolds onto which other sequences, for example, recognition peptides for receptors or other macromolecules, can be grafted. Such constructions could yield chimeras with novel recognition properties. For example, recognition peptides may be linked to a noncovalent coiled coil dimer core to produce a bivalent structure reminiscent of an antibody. The homodimer design provides a means to form bivalent binding molecules with a potential increase of the binding affinity by the square of the K_a (Eilat & Chaiken, 1979; Vinson *et al.*, 1989). Heterodimer chimeric proteins, formed with a heterodimer coiled coil, could be formed to carry two different functions, such as two different binding specificities or a binding domain combined with a protease domain in order to target destruction of a cell or protein. In addition, it may be possible to control the formation of parallel versus antiparallel alignments of coiled coil dimers by charge distribution in the border residues. For example, we currently are investigating the design and characterization of an intramolecular coiled coil stem loop, containing an antiparallel coiled coil dimer linked covalently by a peptide loop, for presenting loop and helical surface recognition sequences (Myszka & Chaiken, 1993). It remains for future investigations to explore such constructs of simplified coiled coil motifs for the *de novo* design of recognition molecules.

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