A Designed Heterotrimeric Coiled Coil†

Shivani Nautiyal,* Derek N. Woolfson,** David S. King,** and Tom Alber*,***

Department of Molecular and Cell Biology, 229 Stanley Hall, University of California, Berkeley, California 94720-3206, and Howard Hughes Medical Institute, 401 Barker Hall, University of California, Berkeley, California 94720-3202

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ABSTRACT: Principles that guide folding of coiled coils were tested by designing three peptides that preferentially associate with each other to form a heterotrimeric coiled coil. The core positions of the designed helices contained residues that promote formation of trimeric coiled coils. Ionic interactions were employed to mediate heterospecificity, and negative design was used to favor formation of the heterotrimer over alternative arrangements. A program was written to select sequences that maximized the number of attractive interhelical interactions in a parallel heterotrimer and the number of repulsive electrostatic interactions in alternative species. Solution studies indicate that an equimolar mixture of the three peptides forms a helical trimer with high specificity and stability. These results validate the principles used to guide the design and suggest that the heterotrimer may serve as a useful, autonomous trimerization domain.

De novo protein design offers an opportunity to define the interactions that determine protein folding. In addition to the challenge of choosing a polypeptide sequence that will adopt a target fold, current work centers on the problem of imparting structural specificity to designed proteins. Target structures have generally been limited to simple motifs such as isolated α-helices (Ihara et al., 1982; Marqusee et al., 1989), four helix bundles (Regan & DeGrado, 1988; Hecht et al., 1990; Handel et al., 1993; Kamtekar et al., 1993), β-sheet proteins (Hecht, 1994; Quinn et al., 1994), and α/β proteins (Tanaka et al., 1994). Coiled coils were identified early as ideal candidates for protein design. On the basis of sequence patterns that were observed in fibrous proteins such as tropomyosin, Hodges and co-workers constructed the first nonbiological coiled coil peptides (St-Pierre & Hodges, 1976; St-Pierre & Hodges, 1977; Hodges et al., 1981). Designed and naturally occurring coiled coils have served as model systems for studying various aspects of protein folding. Progress has been made in understanding the principles that determine the specificity of pairing of coiled coils as well as the determinants of oligomerization order.

Coiled coils are simple structures consisting of two or more amphipathic helices that wrap around each other with a slight supercoil. Coiled-coil sequences contain a characteristic heptad repeat, (a b c d e f g)n, in which the a and d positions are occupied primarily by hydrophobic amino acids. The hydrophobic interface between the helices is formed by residues at positions a, d, e, and g (Crick, 1953; O'Shea et al., 1991; Lovejoy et al., 1993; Harbury et al., 1993, 1994). Formation of salt bridges has been observed between residues at the g position and the e position of the succeeding heptad on the adjacent helix (Figure 1).

Residues at the a and d positions have profound effects on the oligomerization states of coiled coils (Harbury et al.,
FIGURE 1: Schematic side view of a trimeric coiled coil. Thick hatched lines join residues at the e and g positions that can participate in interhelical ion pairs.

1993, 1994; Zhu et al., 1993). Concerted changes in the hydrophobic core of the GCN4 leucine zipper, for example, cause a switch between two-, three-, and four-stranded coiled-coil structures. The switch is governed by packing constraints imposed by distinct local geometries at the a and d positions. The presence of isoleucines at the a positions and leucines at the d positions favors formation of dimers, while the converse arrangement, isoleucines at the d positions and leucines at the a positions, promotes formation of tetramers. When the a and d positions are occupied by isoleucines, a trimeric species is formed. Polar residues at a positions also help determine oligomerization order, with asparagine and lysine favoring dimers and glutamine favoring trimers (L. Gonzalez, Jr., D.N.W. and T.A., unpublished results). These trends are supported by distinct patterns of polar and nonpolar residues at a and d positions of dimeric and trimeric coiled coils (Woolfson & Alber, 1995).

Interhelical electrostatic interactions between g and succeeding e positions can mediate heterospecific association of helices. In the heterodimer formed by the Fos/Jun leucine zipper, association is driven by the destabilization of the Fos homodimer by repulsive interhelical electrostatic interactions that are relieved in the heterodimer (Schueremann et al., 1991; O’Shea et al., 1992). This strategy was used to design obligate heterodimers containing one helix with all the e and g positions occupied by glutamic acids and a second helix with all the e and g positions occupied by lysines (O’Shea et al., 1993; Zhou et al., 1994). The homodimers of each peptide were completely destabilized, presumably by repulsive g to e interactions. The repulsive interactions were relieved when the two peptides were combined, resulting in the formation of stable, heterodimeric coiled coils. In addition, interhelical g to e interactions have been used to predict and design dimerization partners for the bZIP class of DNA binding proteins (Vinson et al., 1993). Finally, the specificity of chain assembly in the heterotrimERIC laminin aminiosigns may be mediated by g to e interactions in the trimeric laminin domain (Beck et al., 1993).

We demonstrate here that interhelical g to e interactions can promote formation of a heterotrimERIC coiled coil. Unlike the heterodimer in which it is straightforward to maximally destabilize the homodimers and maximally stabilize the heterodimer, the case of the heterotrimer is more complicated. For the heterotrimer, it is necessary to destabilize not only homooligomers but also species formed by pairwise association of the helices. The possibility of forming an antiparallel heterotrimer also must be dis favored. To promote formation of the heterotrimer over other possible species, we employed a strategy of negative design. Attractive g to e ion pairs were maximized in the heterotrimer and repulsive interactions were maximized in alternative arrangements. Isoleucines and a single glutamine were placed at the a and d positions of the designed helices to favor the formation of trimers over dimers and tetramers. Our studies show that the three designed peptides preferentially interact with one another to form a stable, heterotrimERIC coiled coil.

EXPERIMENTAL PROCEDURES

Design Rationale. The peptides in the heterotrimer were chosen to be 4.5 heptads long. This is slightly longer than the minimum length required to form an autonomous coiled coil (Lau et al., 1984; Su et al., 1994; Lumb et al., 1994). For a trimeric coiled coil which is 4.5 heptads long, a maximum of 12 g to e interhelical ionic interactions can form. A program was written to maximize the number of favorable ion pairs in the heterotrimer and maximize the number of unfavorable ion pairs in some of the other potential species. Starting with all the possible arrangements of positive and negative charges at the g and e positions, arrangements that would form six or more repulsive g to e interactions in a homotrimer were selected. These charge arrangements were mixed in all possible three-way combinations, and combinations that resulted in 12 favorable g to e interactions were selected. The favorable three-way combinations were screened to maximize the number of repulsive ion pairs if one of the helices was antiparallel. In the antiparallel trimers, residues at the g positions were assumed to contact nearby g residues of the adjacent helices, and, similarly, e residues were assumed to contact nearby e residues in neighboring helices (Lovejoy et al., 1993).

Peptide Synthesis and Purification. Three peptides with the sequences Ac-AEIAIEYEQAAIKKEIAIKDKIAAIKKEYIAAI-Am, Ac-EKIAIEKQAAIEIQKEIAIKDEIAIAM, and Ac-AEIAIEKQAAAIAEKQEIAAEQMIAAI-Am were synthesized on Fmoc1 Rink amide MBHA resin using solid-phase methodology with an Applied Biosystems 431A automated synthesizer. Fmoc-amino acids were activated with HBTU in the presence of HOBT and DIEA; user-devised 1–2 h single coupling cycles employed a 10–15-fold molar excess of acylating species. Protecting groups were Arg(2,2,5,7,8-pentamethylichroman-sulfonyl), Asn(trityl), and Glu(trityl). All peptides were acetylated at the amino terminus. Peptide resin cleavage and peptide deprotection were accomplished in a single step using reagent K (King et al., 1990) for 4 h at 20 °C to afford crude peptide amide. Crude peptides were dissolved in 0–20% acetonitrile/0.1% TFA and purified by reversed-phase HPLC on a Vydac semipreparative (1.0 × 25 cm) C18 column using linear gradients of acetonitrile in water, both containing 0.1% TFA. Peptide purity was greater than 95% as judged by analytical HPLC and electrospray ionization-mass spectrometry (esi-ms) with a Hewlett-Packard 5989A mass spectrometer. All peptides were found to be within 1 Da of the expected mass by esi-ms. Peptide concentrations were determined by tyrosine absorbance in 6 M Gdn-Cl assuming an extinction coefficient of 1280 at 280 nm (Edelhoch, 1967).

Circular Dichroism (CD) Spectroscopy. CD studies were performed with an Aviv Model 62DS spectrometer. Spectra were measured at 10 °C in 150 mM KCl and 50 mM

1 Abbreviations: Ac, acetyl; Am, amide; CD, circular dichroism; esi-ms, electrospray ionization–mass spectrometry; Fmoc, 2-fluorenylmethoxycarbonyl; Gdn-Cl, guanidine chloride; HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid; Tris-Cl, tris-(hydroxymethyl)aminomethane chloride.
potassium phosphate, pH 7.1, using a total peptide concentration of 10 μM. Percent helicity was calculated using a \(\{\theta\}_{222}\) value of 33 000 deg cm² dmol⁻¹ for a fully helical peptide. Thermal denaturations were performed under the same conditions unless otherwise indicated. In melts of the heterotrimer, each peptide was present at a concentration of 3.3 μM. For melts done with combinations of two peptides, each indicated peptide was present at a concentration of 5 μM. Thermal denaturations were performed by monitoring the CD signal at 222 nm as a function of temperature. Temperature was increased in steps of 2 °C with an equilibration time of 2 min and a data averaging time of 1 min. The apparent \(T_m\) values were determined by finding the minimum of the first derivative of CD signal with respect to temperature⁻¹. All melts were greater than 93% reversible.

**Analytical Ultracentrifugation.** Apparent molecular weights of the peptides in solution were determined at 10 °C by sedimentation equilibrium using a Beckman XL-A centrifuge. Experiments were done in 150 mM KCl and 50 mM potassium phosphate, pH 7.1, at total peptide concentrations of 20, 100, and 250 μM. Samples were dialyzed against the indicated buffer for at least 12 h before each experiment. Rotor speeds of 20 000 and 30 000 rpm were used, and data were collected at 229, 276, and 285 nm. Partial specific volumes and solvent densities were determined as described (Laue et al., 1992). Ten to fifteen data sets were fit simultaneously to a single molecular weight using the program HID-4000 (Johnson et al., 1981).

**Identification of Major Solution Species by Ion-Exchange Chromatography.** The individual peptides were mixed in roughly equimolar ratios to a final peptide concentration of 250 μM, and the mixture was subjected to high resolution ion-exchange chromatography on a Vydac 301VHP552 anion-exchange column. The solution species were separated using a linear gradient in which buffer A was 20 mM Tris-Cl, pH 7.3, and buffer B was 20 mM Tris-Cl, pH 7.3, 0.5 M NaCl. The major peaks from several runs were collected, pooled, and dialyzed against 10 mM Tris-Cl, pH 7.3. The dialyzed sample was lyophilized, dissolved in a small volume of H₂O, and diluted to a final peptide concentration of 200 μM in 6 M urea. The samples were allowed to equilibrate for 45 min and then dialyzed against 20 mM Tris-Cl, pH 7.3 for >36 h. The dialyzed mixtures were chromatographed again using ion-exchange chromatography as described above. The components of the peak fractions were determined by analytical reversed-phase HPLC using a linear H₂O/acetonitrile gradient in the presence of 0.1% TFA.

**Native Gel Electrophoresis.** The three individual peptides, pairwise mixtures of the peptides, and an equimolar mixture of all three peptides were analyzed by native gel electrophoresis. The gels were 7.5% polyacrylamide and 375 mM Tris-Cl, pH 8.9, and the running buffer was 25 mM Tris-glycine, pH 8.5. Samples were prepared in 50 mM Tris-Cl, pH 7.3, at a total peptide concentration of 250 μM and diluted 2-fold with sample loading buffer containing 750 mM Tris-Cl, pH 8.9, 20% glycerol, and 0.1% bromophenol blue (w/v) before being loaded onto the gel. Gels were fixed with 2% glutaraldehyde before staining with Coomassie blue.

**RESULTS**

Helices 21–28 residues in length are sufficient to form an autonomous coiled coil (Lau et al., 1984; Su et al., 1994; Lumb et al., 1994). To confer adequate stability, we made the heterotrimer 34 residues long. To promote trimer formation, isoleucines were placed at the α and β positions of each peptide. This choice was based on the observation that a mutant of the GCN4 leucine zipper in which eight α and β residues were changed to isoleucines showed a strong preference for forming a trimeric species (Harbury et al., 1993). The polar amino acid glutamine was placed at the second α position of each peptide to destabilize the heterotrimer sufficiently to characterize unfolding in the absence of chemical denaturants. The glutamines were placed in the same α position in all three peptides to avoid burying the polar residues in a hydrophobic context and to restrict the register and direction of the helices. Glutamine was selected because it occurs with a high frequency at the α positions of naturally occurring trimeric coiled coils (Woolfson & Alber, 1995) and because the Asn16Gln mutant of the GCN4 leucine zipper can form trimers (L. Gonzalez, Jr., D.N.W., and T.A., unpublished observations).

Two strategies were used to achieve preferential association of the three peptides: (i) interhelical charge complementation was maximized in the heterotrimer, and (ii) repulsive interhelical interactions were maximized in alternative species. For a trimeric coiled coil that is 4.5 heptads long, a maximum of 12 g to e interhelical ionic interactions can form. If, in each peptide, eight g and e positions can be occupied by a charged residue, there are \(2^8\) or 256 different ways to arrange charges at these positions. Three different charge arrangements can be combined in greater than \(1.6 \times 10^5\) ways (256 × 255 × 254). A program was written to sort through these possibilities to find combinations of charges that optimally fulfilled our design criteria.

The program first selected arrangements of charged residues at the g and e positions that would result in six or more repulsive interactions in a homotrimer. Of the 256 different ways to arrange positive and negative charges at g and e, 176 resulted in 6 or more repulsive interactions in a homotrimer. The 176 charge arrangements were then mixed in all three-way combinations \((5 \times 10^5)\), and combinations that would form 12 favorable g to e interactions in parallel heterotrimers were selected. This produced 208 three-way combinations of oppositely charged residues that maximized charge complementation in a heterotrimer. These were further screened to maximize the number of repulsive ion pairs if one of the helices was antiparallel. This resulted in two distinct arrangements of changes that fit all the criteria. One of these two combinations was used for the design. Each individual designed peptide can form six attractive and six repulsive g to e interactions in a parallel homotrimer. Trimmers containing two of the three peptides (AAB, AAC, ABB, ACC, BCC, and BBC) would have a maximum of eight favorable ionic interactions and four unfavorable ionic interactions between g and e positions of adjacent helices.

Sequences of three peptides, A, B, and C, were chosen to implement the selected pattern of charges (Figure 2). Glutamic acid and lysine were used as the negatively and positively charged residues at the g and e positions. Alanines were placed at the α and β positions of each peptide to reduce intrahelical interactions of charged residues at adjacent e and g positions and to help promote helix formation. Glutamines were incorporated at exterior positions 18 and 33 of the B peptide to help alleviate solubility problems. Tyrosines were included to provide chromophores for concentration deter-
peptides and an equimolar mixture of A, B, and C gave by analytical ultracentrifugation. The individual B and C peptides were approximately 80, 70, and 90% helical, respectively, while an equimolar mixture of the three peptides was determined by high-resolution ion-exchange chromatography. To permit separation on the basis of charge, the net charges of the A, B, and C peptides were selected to be -3, -4, and 0 for the respective monomers (-9, -12, and 0 for the homotrimers) and -7 for the heterotrimer. Analysis of an equimolar mixture of A, B, and C by high-resolution ion-exchange chromatography yielded one major peak and several minor peaks (Figure 4A, top chromatogram). Reversed-phase chromatography indicated the major peak was composed of a 1:1:1 ratio of A, B, and C (data not shown). To determine whether the minor peaks arose from mixing errors or from low specificity, the purified heterotrimer was denatured in 6 M urea, renatured, and analyzed again by ion-exchange chromatography. Only one peak was detected (Figure 4A, middle chromatogram). The peak had a retention time distinct from that of the individual A, B, and C peptides. Analytical reversed-phase HPLC of this peak showed three peaks that corresponded to the A, B, and C peptides (Figure 4B). Integration of these peaks and normalization for the extinction coefficients revealed a 1:1:1 stoichiometry of A, B, and C (Figure 4B).

Native gel electrophoresis at pH 8.5 also was used to visualize the species formed by an equimolar mixture of the three peptides (Figure 5). The individual A, B, and C peptides, pairwise mixtures of the peptides, and an equimolar mixture of all three peptides purified by ion-exchange chromatography (Figure 4A, middle chromatogram) were analyzed. The A, B, and C peptides migrate as distinct species. The A and B mixture gave one major band and one minor band with mobilities similar to those of the A and B peptides. The mixture of A, B, and C showed two distinct bands, and the mixture of B and C produced two smeared bands. The equimolar mixture of A, B, and C showed only one band (Figure 5).

Thermal denaturation studies indicated that the heterotrimer is more thermally stable than all the alternative species. The A and C peptides had apparent Tₘ's of 50.5 and 58.5 °C, respectively (Figure 6 and Table 1). The B peptide showed a broad, noncooperative melting transition. In contrast, an equimolar mixture of A, B, and C melted molecular weights consistent with trimeric conformations (Table 1). The A peptide had an apparent molecular mass of 13,820 Da, which is intermediate between the weight expected for a trimer and a tetramer, and showed significant systematic deviations from the determined molecular mass (data not shown).

The distribution of solution species formed in the equimolar mixture of the three peptides was assessed by ion-exchange chromatography. To permit separation on the basis of charge, the net charges of the A, B, and C peptides were given a different net charge to allow separation of solution species by ion-exchange chromatography and native gel electrophoresis. Charged or polar residues were placed at the f positions to help increase solubility. In addition, the overall net charge of each peptide was modulated by varying the positions to help increase solubility. In addition, the overall net charge of each peptide was modulated by varying the positions to help increase solubility. In addition, the overall net charge of each peptide was modulated by varying the positions to help increase solubility.

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Figure 4: Ion-exchange chromatography of the heterotrimer. (A) The three peptides were combined in approximately equimolar ratios and analyzed using high-pressure ion exchange chromatography (top chromatogram). Examination of the major peak by analytical reverse-phase HPLC revealed that A, B, and C were present in a 1:1:1 stoichiometry (not shown). The peak sample was denatured with 6 M urea, renatured, and analyzed again using ion exchange chromatography (middle chromatogram). Only one peak was detected. This peak has a retention time which is distinct from that of the individual A, B, and C peptides (bottom chromatogram). (B) Analytical reversed-phase HPLC of the major peak shown in the middle chromatogram of 4A showed a 2:1:1 ratio of peak area for A:B:C, corresponding to a 1:1:1 ratio of A:B:C after normalization for the extinction coefficients.

To probe the mechanism of specificity, the effects of increasing ionic strength on the thermal denaturation of each peptide were measured. Upon increasing the KCl concentration from 150 to 500 mM, the apparent $T_m$'s of A and C increased by 5.5 and 6.0 °C, respectively, and the melting transition of the B peptide remained broad and noncooperative. In contrast, no change in apparent $T_m$ was observed for the ABC mixture.

**DISCUSSION**

Using principles of folding of coiled coil proteins, three peptides that preferentially interact with each other to form a stable, heterotrimeric coiled coil were designed. Trimerization of the peptides was mediated by placing isoleucines at the a and d positions of all the peptides. This pattern has been shown to promote trimerization of a GCN4 leucine zipper variant (Harbury et al., 1993, 1994). Negative design was used to favor heterotrimerization. Formation of the heterotrimer was favored by maximizing the number of attractive g to e interhelical interactions in the heterotrimer, while at the same time maximizing repulsive electrostatic
interactions in alternative species. Homotrimers and anti-parallel heterotrimers were specifically selected against in this new design approach.

Solution studies indicated that our design strategy was successful. A, B, and C interact specifically to form a helical, trimeric species. An equimolar mixture of the three peptides showed more helical structure than any of the individual peptides or their pairwise combinations (Figure 3 and Table 1). Examination of an equimolar mixture of the three peptides by ion-exchange chromatography (Figure 4) revealed only the heterotrimERIC species. Given the sensitivity of detection, these results indicate that the heterotrimer is favored by at least 100-fold over other combinations. Further analysis by native gel electrophoresis revealed that the heterotrimer migrated as one band distinct from the individual peptides and all pairwise combinations (Figure 5).

In addition to having high specificity, the heterotrimer was the most thermally stable species. The thermal melt of the heterotrimer was cooperative (Figure 6), as observed for typical globular proteins, with an apparent $T_m$ over 85 °C (Table 1). None of the individual peptides or pairwise combinations had apparent $T_m$'s comparable to that of the heterotrimer, further supporting preferential association of the three peptides. Crystals of the heterotrimer that diffract X-rays to 2.0 Å resolution have been obtained (data not shown). The growth of well ordered crystals is consistent with forming of a relatively unique native structure. The crystal structure is currently being determined to elucidate the orientation of the helices and to assess whether the specific tertiary interactions that form the basis of the design are made.

While it appears that electrostatic interactions between residues at the g and e positions are mediating association of the three helices, the energetic basis for heterospecificity remains to be determined. The stabilization of the individual peptides caused by increasing ionic strength, coupled with the absence of salt effects on the heterotrimer, suggests that heterotrimer formation is driven at least in part by unfavorable interhelical electrostatic interactions in the homotrimers that are relieved upon heterotrimer formation. This mechanism also mediates dimerization specificity by both the Fos/Jun leucine zipper (O’Shea et al., 1992) and Velcro, a designed heterodimeric coiled coil (O’Shea et al., 1993). NMR studies (Lumb & Kim, 1995) and theoretical calculations (Hendsch & Tidor, 1994) indicate that g to e ion pairs contribute little to the thermodynamic stability of dimeric coiled coils. In some cases, g to e ion pairs may actually be neutral or destabilizing.

The free energy contribution of a salt bridge, however, may vary depending on the neighboring core residues and on the oligomeric state of the coiled coil. Comparison of the crystal structure of the wild-type GCN4 leucine zipper with a trimeric variant indicates that interhelical g to e salt bridges are more common in the trimer than in the dimer (Harbury et al., 1994). The salt bridges in the trimer are also shorter and more buried than those in the dimer. These observations suggest the possibility that attractive g to e, Lys-Glu ion pairs in trimeric coiled coils may contribute to thermodynamic stability and may drive heterospecific association.

In addition to testing principles of protein folding, protein design can generate molecules with practical utility. Protein chimeras of coiled coils with other functional domains—including the lambda repressor and lexA DNA-binding domains (Hu et al., 1990; Schmidt-Dörr et al., 1991), T-cell receptor (Kostelny et al., 1992), single chain Fv fragments of antibodies (Pack et al., 1995), and maltose binding protein (Blondel & Bedouelle, 1991)—have been reported. The stability and specificity of the ABC heterotrimer suggest that it can be used as an autonomous oligomerization domain for mediating association of three different proteins.

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