A Buried Polar Interaction Imparts Structural Uniqueness in a Designed Heterodimeric Coiled Coil†

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ABSTRACT: Buried polar residues are a common feature of natural proteins. ACID-p1 and BASE-p1 are two designed peptides that form a parallel, heterodimeric coiled coil with a fixed tertiary structure [O'Shea, E. K., Lumb, K. J., & Kim, P. S. (1993) *Curr. Biol. 3*, 658−667]. The interface between the ACID-p1 and BASE-p1 helices consists of hydrophobic Leu residues, with the exception of a single polar residue, Asn 14. In the crystal structure of the GCN4 leucine zipper coiled coil, an analogous Asn is hydrogen bonded to the corresponding Asn of the opposing helix, thereby forming a buried polar interaction in an otherwise hydrophobic interface between the helices [O'Shea, E. K., Klemm, J. D., Kim, P. S., & Alber, T. (1991) *Science 254*, 539−544]. This buried polar interaction in the ACID-p1/BASE-p1 heterodimer was removed by substituting Asn 14 with Leu. The Asn 14→Leu variants are significantly more stable than the p1 peptides and preferentially form a heterotetramer instead of a heterodimer. Strikingly, the heterotetramer does not fold into a unique structure; in particular, the helices lack a unique orientation. Thus, the Asn 14 residue imparts specificity for formation of a two-stranded, parallel coiled coil at the expense of stability. The results suggest that, whereas nonspecific hydrophobic interactions contribute to protein stability, the requirement to satisfy the hydrogen bonding potential of buried polar residues in the generally hydrophobic environment of the protein interior can impart specificity (structural uniqueness) to protein folding and design.

Globular proteins are typified by a hydrophobic interior comprising mostly apolar residues, with polar residues generally located on the protein surface (Kauzmann, 1959; Dill, 1990). The burial of a polar residue in the hydrophobic interior of a protein is destabilizing relative to substitution by a hydrophobic isostere (Sauer & Lim, 1992). Nonetheless, buried polar residues are a general feature of proteins (Barlow & Thornton, 1983; Rashin & Honig, 1984) and increasingly appear to be important determinants of conformational specificity in protein folding (Chothia, 1976; O'Shea et al., 1991, 1993; Harbury et al., 1993; Hendsch & Tidor, 1994; Betz et al., 1995).

The coiled coil is a widespread structural motif formed from two or more α-helices (Cohen & Parry, 1994). The sequences of coiled coils are characterized by a heptad repeat of seven amino acid residues denoted **a** to **g** (McLachlan & Stewart, 1975). Most of the residues at positions **a** and **d** are hydrophobic, forming the characteristic 4−3 repeat of coiled coils, and residues at positions **e** and **g** are often charged (Hodges et al., 1972; McLachlan & Stewart, 1975; Parry, 1982; Conway & Parry, 1990; Lupas et al., 1991; Berger et al., 1995). The hydrophobic interface between the α-helices is formed by residues at positions **a**, **d**, **e**, and **g**, and the side chains of the **e** and **g** residues can also participate in interhelical electrostatic interactions [O'Shea et al., 1991; Harbury et al., 1993, 1994; Glover & Harrison, 1995; see also Lumb and Kim (1995)].

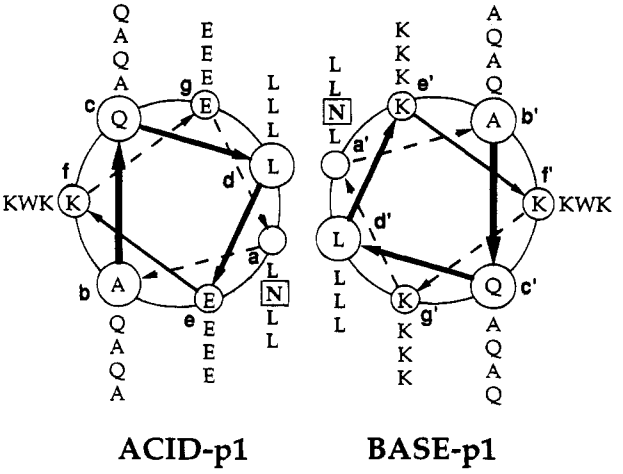

FIGURE 1: Helical wheel representation of the ACID-p1/BASE-p1 heterodimer (O'Shea et al., 1993). The **a** and **d** interface residues are Leu, with the single exception of Asn 14 at position **a** (boxed residue). In the ACID-pLL and BASE-pLL peptides, Asn 14 is substituted for Leu. BASE-p1 differs from ACID-p1 only at positions **e** and **g**, where ACID-p1 contains Glu and BASE-p1 contains Lys. The homodimers are destabilized at neutral pH by unfavorable interhelical interactions between residues at positions **e** and **g**, and this destabilization is relieved in the heterodimer (O'Shea et al., 1993).

Peptide "Velcro" is a designed heterodimeric, parallel, two-stranded coiled coil (Figure 1; O'Shea et al., 1993) that has been used to bring together two different proteins *in vivo* (Chang et al., 1994). The two peptides (ACID-p1 and BASE-p1) form a stable heterodimeric coiled coil, but the individual peptides do not form stable homodimers at neutral pH (O'Shea et al., 1993). The ACID-p1/BASE-p1 het-

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erodimer has a dissociation constant of 30 nM at 20 °C in PBS, pH 7. ACID-p1 and BASE-p1 have identical sequences, except for the residues at the **e** and **g** positions, which are Glu in ACID-p1 and Lys in BASE-p1 (Figure 1). The pH and ionic strength dependence of stability and pK_a measurements indicate that the heterodimerization specificity results predominantly from electrostatic destabilization of the homodimers (related strategies have been described; Graddis et al., 1993; Zhou et al., 1994). Hydrogen exchange studies indicate that the ACID-p1/BASE-p1 heterodimer has a well-packed hydrophobic core (O'Shea et al., 1993), in contrast to other designed proteins that have been carefully characterized (DeGrado et al., 1991; Raleigh & DeGrado, 1992; Richardson et al., 1992; Betz et al., 1993; Handel et al., 1993; Quinn et al., 1994; Tanaka et al., 1994a,b).

The ACID-p1 and BASE-p1 peptides have Leu residues at the **a** and **d** positions in the hydrophobic interface, with the exception of a single buried polar residue, Asn 14, at an **a** position (Figure 1). In the crystal structure of the GCN4 leucine zipper, a parallel, two-stranded coiled coil, an analogous Asn is hydrogen bonded to the corresponding Asn of the opposing helix (O'Shea et al., 1991). The preservation of this buried polar interaction was an explicit feature of the design of the ACID-p1/BASE-p1 heterodimer (O'Shea et al., 1993) and was intended to promote a parallel, unstaggered orientation of two helices (O'Shea et al., 1991; Harbury et al., 1993).

We substituted Asn 14 with Leu (N14L) to evaluate the contribution of this single buried polar residue to the properties of the designed ACID-p1/BASE-p1 heterodimer. We find that the N14L variants form a heterotetramer that lacks a unique arrangement of helices. Thus, Asn 14 imparts structural uniqueness to the ACID-p1/BASE-p1 heterodimer. The results support the notion that hydrophobic interactions contribute stability, but not necessarily specificity, to protein structure, whereas buried polar interactions introduce structural uniqueness.

MATERIALS AND METHODS

Peptides were synthesized by solid-phase Fmoc methods and purified by reversed-phase HPLC[1] as described previously (O'Shea et al., 1993). The identity of each peptide was confirmed by laser desorption mass spectrometry on a Finnigan Lasermat. For each peptide, the expected and observed molecular masses agreed to within 2 Da. All peptides were acetylated at the N terminus and amidated at the C terminus. Disulfide-bonded peptides were produced by air oxidation of the reduced peptides in 6 M GdnHCl and 0.2 M Tris-base, pH 8.7, and purified by reversed-phase HPLC as described previously (O'Shea et al., 1993).

CD spectroscopy was performed with an Aviv 62DS spectrometer. Peptide concentrations were determined by Trp absorbance in 6 M GdnHCl at 25 °C using an extinction coefficient at 280 nm of 5690 M^{-1} cm^{-1} (Edelhoch, 1967). T_m values were measured at a total peptide concentration (monomer) of 10 μM in PBS (10 mM sodium phosphate, 150 mM NaCl) or in 50 mM Tris-base, 150 mM NaCl, and 2.0 M GdnHCl, pH 8.7, as described previously (Lumb et al., 1994). Thermal melts were reversible (the forward and reverse melts were superimposable, with greater than 95% of the starting signal regained on cooling). $\Delta G°$ values in PBS (25 °C, pH 7.0) were estimated from a linear extrapolation of the dependence of ΔG on urea concentration in the transition region for samples containing 20 μM total peptide (Tanford, 1970; Pace, 1986). Urea (Nacalai Tesque, Inc., Kyoto, Japan) concentration was determined by measurements of refractive index (Nozaki, 1972).

Apparent molecular masses were determined at 25 °C by sedimentation equilibrium with a Beckman XL-A ultracentrifuge. Samples were dialyzed against the reference buffer (PBS, unless specified otherwise). At least three different sample concentrations, corresponding to initial absorbance values at 280 nm in the range 0.1−1, were spun at speeds in the range 30−50 krpm in an An-60Ti rotor. Data were analyzed using Origin (Beckman Instruments, Palo Alto, CA). Discrimination between different equilibria (e.g., single species or monomer−dimer) was based on the distribution of residuals. In all cases, the random distribution of residuals indicates that the data fit well to the appropriate model. Partial molar volumes, solvent densities, and the effects of GdnHCl were calculated as described by Laue et al. (1992).

Helix orientation was determined using variants of the ACID-pLL and BASE-pLL peptides with additions of Ac-Trp-Cys-Gly-Gly to the N terminus (denoted ACID-pLL^N and BASE-pLL^N) or Gly-Gly-Cys-$CONH_2$ to the C terminus (denoted ACID-pLL^C and BASE-pLL^C). The two Gly residues provide flexibility for disulfide bond formation, and the additional Trp of the N-terminal variants facilitates HPLC separation of the peptides [cf. Harbury et al. (1993)]. Peptides (10 μM total monomer concentration) were incubated at 25 °C in redox buffer (250 mM oxidized glutathione, 500 mM reduced glutathione, 50 mM Tris-base, 150 mM NaCl, 2 M GdnHCl, 0.1% NaN_3, pH 8.7) in an anaerobic chamber to allow disulfide bond formation and exchange of peptide monomers. The reaction was quenched with acetic acid (10% final concentration). The products were analyzed by reversed-phase HPLC on an analytical C_{18} column (Vydac) using a linear water/acetonitrile gradient containing 0.1% trifluoroacetic acid. Peptides were detected by absorbance at 280 nm.

^{1}H NMR spectroscopy was performed at 500.1 MHz with a Bruker AMX spectrometer. Samples were 1.0 mM (total peptide concentration) in PBS, pH 7.0, and internally referenced to zero ppm with trimethylsilylpropionic acid. One-dimensional data sets were acquired at 25 °C using a spectral width of 6024.1 Hz and solvent presaturation. Data sets were defined by 4096 complex points and zero filled once before Fourier transformation. Hydrogen exchange studies were initiated by dissolving lyophilized samples in D_2O. pH readings in D_2O were uncorrected for the isotope effect.

The observed hydrogen exchange rates of the slowest exchanging protons were compared to the rates predicted by assuming that global unfolding is required for proton exchange. For an oligomeric protein $K = (nc^{n-1}f_u^n)/(1 - f_u)$, where K is the equilibrium constant for unfolding, n is

[1] Abbreviations: $\Delta G°$, free energy of unfolding at a total peptide concentration of 1 M; $[\theta]_{222}$, molar ellipicity at 222 nm; k_{ex}, observed amide exchange rate; k_{int}, rate of amide exchange in a random coil polypeptide; ANS, 1-anilinonaphthalene-8-sulfonic acid; CD, circular dichroism; f_u, fraction unfolded; GdnHCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; K_d, dissociation constant; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline (10 mM sodium phosphate, 150 mM NaCl); ppm, parts per million; T_m, midpoint of the thermal unfolding transition.

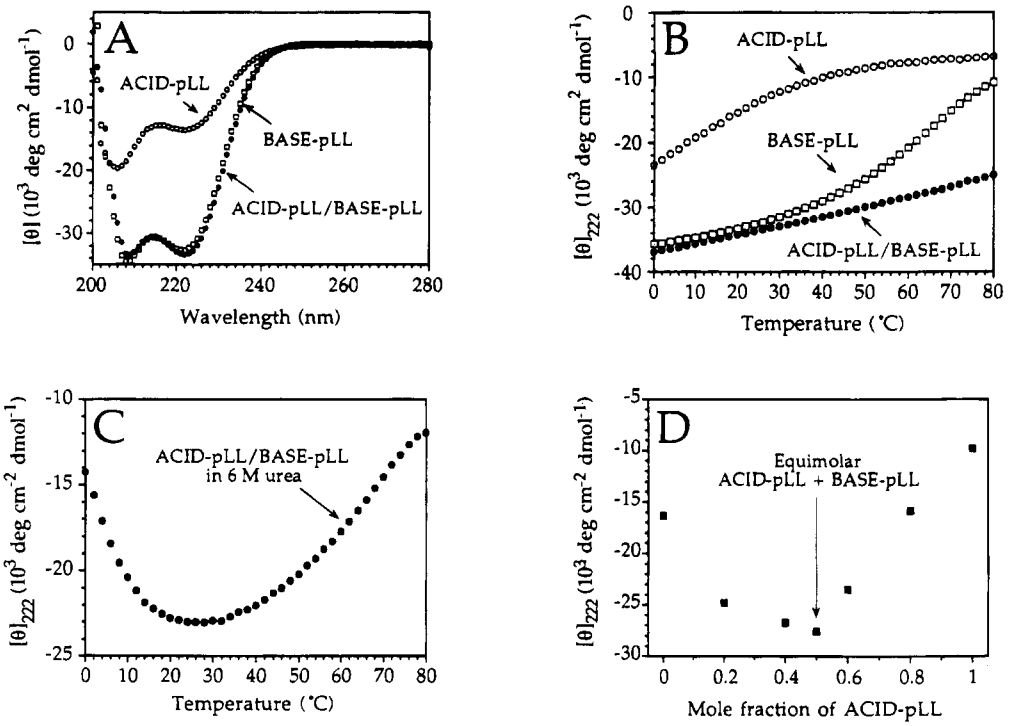

FIGURE 2: (A) CD spectra of ACID-pLL, BASE-pLL, and an equimolar mixture of ACID-pLL and BASE-pLL (PBS, 25 °C, pH 7.0, total peptide concentration 10 μM). The minima at 208 and 222 nm indicate that the peptides form helical structures. The CD spectrum of an equimolar mixture of ACID-pLL and BASE-pLL is not the average of the spectra of the isolated ACID-pLL and BASE-pLL peptides, indicating that the peptides associate to form a heterospecies (ACID-pLL/BASE-pLL). (B) Temperature dependence of $[\theta]_{222}$ for ACID-pLL, BASE-pLL, and an equimolar mixture of ACID-pLL and BASE-pLL (PBS, pH 7.0, total peptide concentration 10 μM). ACID-pLL and BASE-pLL show thermal unfolding transitions with T_m values of ~20 and ~64 °C, respectively. When mixed, the two peptides associate to form a new species (ACID-pLL/BASE-pLL) with a T_m greater than 100 °C. (C) In 6 M urea (PBS, pH 7.0, total peptide concentration 10 μM), ACID-pLL/BASE-pLL exhibits both low- and high-temperature unfolding transitions, similar to the properties of the designed four-helix bundle α_4 (Handel et al., 1993). (D) The stoichiometry of the ACID-pLL and BASE-pLL peptides in the heterotetramer is 1:1. The helicity of the mixture as measured by $[\theta]_{222}$ is maximal when ACID-pLL and BASE-pLL are mixed in equimolar amounts (PBS, 70 °C, pH 7.0, total peptide concentration 10 μM).

the oligomerization order, c is the total peptide concentration, and f_u is the fraction of unfolded (monomeric) peptide. When exchange occurs only from the globally unfolded protein, and the folded protein is stable ($f_u \ll 1$), the predicted protection factor (k_{int}/k_{ex}) is, in principle, equal to $1/f_u$ (where k_{int} and k_{ex} are the random-coil and observed hydrogen exchange rates, respectively; Englander & Kallenbach, 1984; Bai et al., 1994). For naturally occurring globular proteins, the slowest exchanging amide protons often have exchange rates an order of magnitude slower than predicted from f_u (Englander & Kallenbach, 1984; Roder, 1989; Loh et al., 1993; Mayo & Baldwin, 1993; Bai et al., 1994). An average intrinsic exchange rate of 31 s^{-1} was used for k_{int}, corresponding to 3 times the rate of amide exchange for poly-DL-alanine at pH 7.0 and 25 °C (Englander et al., 1972). The predicted protection factor for a 3 mM solution of the ACID-p1/BASE-p1 heterodimer was erroneously reported to be 10^5 in O'Shea et al. (1993), instead of 10^4 (see Figure 6A legend).

Fluorescence studies of binding to ANS (Molecular Probes, Inc., Eugene, OR) were performed with an ISS GREG PC fluorometer at 25 °C. Samples were prepared in PBS and contained 5 μM ANS. Excitation was at 370 nm. Apparent K_d values were estimated by assuming a single binding site and fitting the change in ANS emission intensity (ΔF) with peptide concentration (c) to the equation $\Delta F = \Delta F_{max} - K_d \Delta F/c$ (Fersht, 1985).

RESULTS

Characterization of the Isolated ACID-pLL and BASE-pLL Peptides. CD spectra indicate that the isolated ACID-pLL and BASE-pLL peptides are helical at neutral pH (Figure 2A) and exhibit thermally induced unfolding transitions (Figure 2B). BASE-pLL is more stable than ACID-pLL, probably due to the longer side chain of Lys, which allows for more flexibility and better solvation of the terminal charged group (O'Shea et al., 1993). BASE-pLL is dimeric at neutral pH by sedimentation equilibrium (BASE-pLL at pH 7.0: observed molecular mass 6.81 kDa; expected for dimer 7.12 kDa), with a $\Delta G°$ (pH 7.0) determined by urea denaturation of −8.1 kcal/mol (m = 0.8 kcal mol^{-1} M^{-1}; unpublished results). For comparison, $\Delta G°$ (pH 7.0) for the BASE-p1 homodimer is approximately −4 kcal/mol (O'Shea et al., 1993). Sedimentation equilibrium data for ACID-pLL at pH 7.0 fit a monomer−dimer−tetramer equilibrium with monomer−dimer and dimer−tetramer K_d values of 0.9 and 0.2 mM, respectively, corresponding to $\Delta G°$ values of −4 and −5 kcal/mol, respectively (unpublished results). For comparison, the upper limit of $\Delta G°$ for the ACID-p1 homodimer at pH 7.0 is −3 kcal/mol (O'Shea et al., 1993). The ACID-pLL and BASE-pLL peptides also form homotetramers at pH 3 and 11, respectively, with T_m values greater than 100 °C (ACID-pLL at pH 3: observed molecular mass 14.0 kDa; expected for tetramer 14.3 kDa. BASE-pLL at pH 11: observed molecular mass 14.4 kDa; expected for tetramer 14.2 kDa).

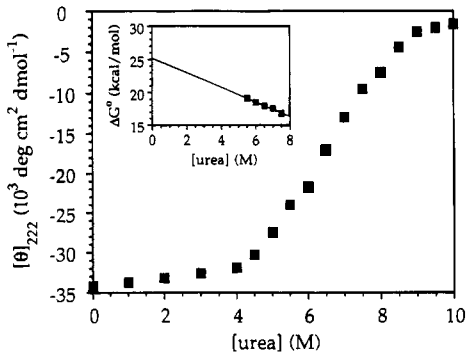

FIGURE 3: Urea denaturation curve of the ACID-pLL/BASE-pLL heterotetramer. The data were fit to a monomer−tetramer equilibrium (inset) to yield an apparent $\Delta G°$ (25 °C, pH 7.0) of −27 kcal/mol (m = 1.2 kcal mol^{-1} M^{-1}). For comparison, $\Delta G°$ (20 °C, pH 7) for the ACID-p1/BASE-p1 homodimer is −10.1 kcal/mol (O'Shea et al., 1993).

ACID-pLL and BASE-pLL Form a Heterotetramer. On mixing, the ACID-pLL and BASE-pLL peptides form a new helical species[2] (Figure 2A) that has a T_m at pH 7.0 of greater than 100 °C (Figure 2B). In 6 M urea, ACID-pLL/BASE-pLL exhibits both low- and high-temperature unfolding transitions, with a temperature of maximum stability of approximately 26 °C (Figure 2C). Sedimentation equilibrium indicates that the ACID-pLL and BASE-pLL peptides form a tetramer in PBS at pH 7.0 (ACID-pLL/BASE-pLL at pH 7.0: observed molecular mass 13.8 kDa; expected for tetramer 14.2 kDa), with an apparent $\Delta G°$ (pH 7.0) determined by urea denaturation of −27 kcal/mol (Figure 3). The helical content monitored by $[\theta]_{222}$ of a mixture of ACID-pLL and BASE-pLL is maximal when the two peptides are mixed in equimolar amounts, indicating that the stoichiometry of the two peptides in the heterotetramer is 1:1 (Figure 2D).

Helix Orientation in the ACID-pLL/BASE-pLL Heterotetramer. Disulfide bond formation was used to determine the orientation of the helices of the heterotetramer [Figure 4A; cf. Harbury et al., (1993)]. Experiments are performed in redox buffer that allows the peptide monomers and disulfide bonds to rearrange. The products are analyzed by reversed-phase HPLC. Sedimentation equilibrium and CD experiments confirm that, under the redox conditions, the ACID-pLL and BASE-pLL peptides still preferentially associate to form a heterotetramer (Figure 5).

The two ACID-pLL helices of the heterotetramer adopt predominantly the antiparallel orientation with respect to each other, with a small fraction of the helices in the parallel orientation (Figure 4B). The two BASE-pLL helices of the heterotetramer can also adopt both orientations with respect to each other (Figure 4C). Neither orientation of the BASE-pLL helices dominates in the heterotetramer. Thus, the ACID-pLL/BASE-pLL heterotetramer does not fold into a structure with a unique orientation of helices.

Hydrogen Exchange Studies. Natural globular proteins typically contain a subset of amide protons with hydrogen exchange rates approximately 10-fold slower than expected if exchange occurred only from globally unfolded molecules (Englander & Kallenbach, 1984; Roder, 1989; Loh et al., 1993; Mayo & Baldwin, 1993; Bai et al., 1994). This feature has proved difficult to incorporate into designed proteins, presumably due to poor packing of side chains or structural fluctuations that are greater than those typically occurring in native proteins (DeGrado et al., 1991; Richardson et al., 1992; Betz et al., 1993).

The most protected amides of the ACID-p1/BASE-p1 heterodimer exchange 10-fold more slowly than expected if exchange occurred via global unfolding (Figure 6A). This result suggests strongly that the ACID-p1/BASE-p1 heterodimer has a well-packed helical interface [see also O'Shea et al. (1993)]. In contrast, the slowest amide proton exchange rates of the ACID-pLL/BASE-pLL heterotetramer are 4 orders of magnitude faster than expected for a global unfolding mechanism (Figure 6B), indicating that the ACID-pLL/BASE-pLL heterotetramer has a fluctuating structure. For comparison, the hydrogen exchange rates of the designed four-helix bundle α_4, which has fluctuating structure (Handel et al., 1993), are 5 orders of magnitude faster than expected if exchange occurred from globally unfolded molecules (DeGrado et al., 1991).

ANS Binding Studies. Molten globules and many designed proteins bind the hydrophobic dye ANS, presumably because these proteins are loosely packed (Goto & Fink, 1989; Semisotnov et al., 1991; Handel et al., 1993). Native proteins bind ANS weakly, if at all, unless there is a solvent-accessible nonpolar region. ANS does not bind appreciably to the ACID-p1/BASE-p1 heterodimer (Figure 7). In contrast, ANS binds weakly to the ACID-pLL/BASE-pLL heterotetramer, but not as avidly as to the molten globule form of α-lactalbumin (Figure 7).

DISCUSSION

A remarkable feature of natural proteins is structural uniqueness. Most naturally occurring proteins adopt a single, well-defined fold with many efficiently packed side chains that exclude solvent. A native level of conformational specificity and efficient side-chain packing has proved both easy to disrupt in natural proteins (Kuwajima, 1989; Dobson, 1994) and difficult to incorporate into designed proteins. Designed proteins often exhibit properties such as linear thermal unfolding transitions, ANS binding, and fast amide proton exchange rates (DeGrado et al., 1991; Raleigh & DeGrado, 1992; Richardson et al., 1992; Betz et al., 1993; Handel et al., 1993; Fezoui et al., 1994; Quinn et al., 1994; Tanaka et al., 1994a,b). These properties suggest that ill-defined structures with loosely packed hydrophobic residues are formed (DeGrado et al., 1991; Richardson et al., 1992). Helical bundles provide the paradigm for achieving limited conformational specificity in protein design. Designed helical bundles, while stable, typically have poorly packed hydrophobic residues (DeGrado et al., 1991; Raleigh & DeGrado, 1992; Handel et al., 1993) or exist in multiple

[2] The avoidance of unfavorable interhelical electrostatic interactions between residues at positions **e** and **g**, as opposed to the formation of putatively favorable interhelical salt bridges, is an important determinant of dimerization specificity in two-stranded coiled coils (O'Shea et al., 1992, 1993; John et al., 1994; Lumb & Kim, 1995). The T_m values of the isolated ACID-pLL and BASE-pLL peptides exceed 100 °C at pH 3.0 and 11.0, respectively, where the side chains of the **e** and **g** residues are neutral, or at pH 7 in 2 M NaCl (which would shield electrostatic interactions), suggesting that the relief of electrostatic destabilization in the homodimers at pH 7 contributes substantially to the specificity of heterotetramer formation. The ACID-pLL/BASE-pLL heterotetramer is too stable to denature thermally in the absence of denaturants (Figure 2B). Studies of the pH dependence of the ACID-pLL/BASE-pLL heterotetramer in 6 M urea are complicated by the presence of multiple unfolding transitions at extremes of pH (unpublished results), precluding an unambiguous assessment of the contribution of interhelical salt bridges to the stability of the heterotetramer.

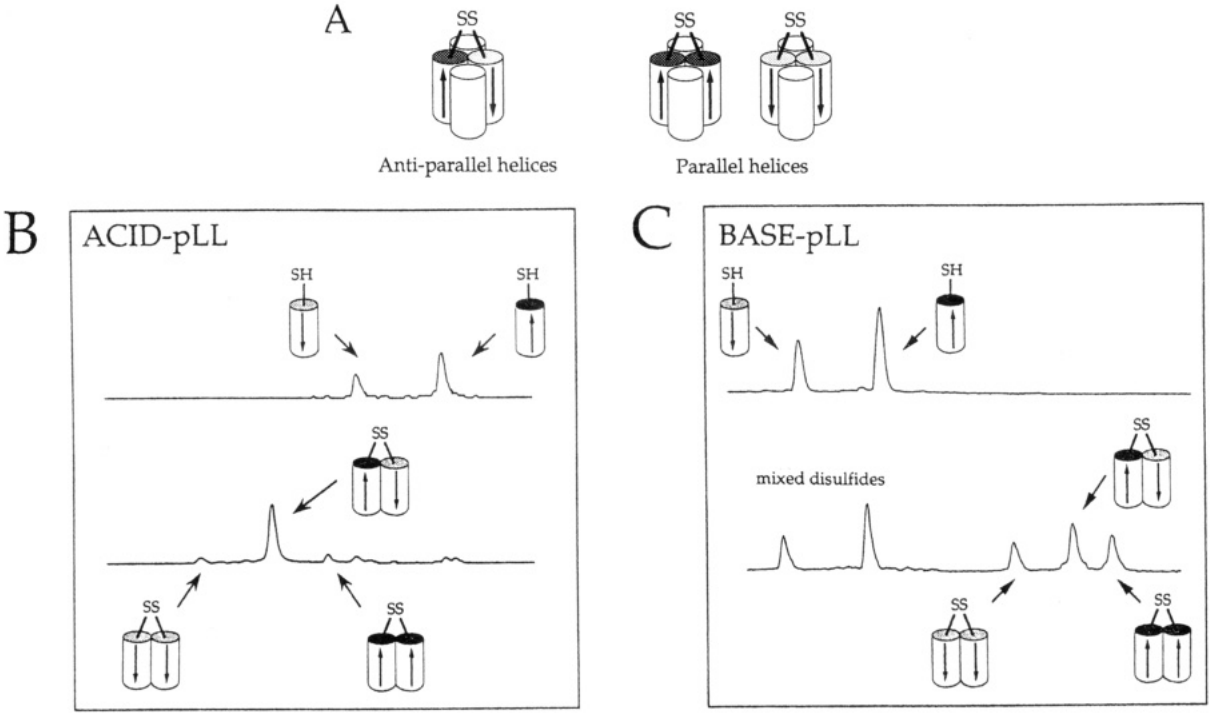

FIGURE 4: (A) Strategy for determining helix orientation in the heterotetramer. Variant ACID-pLL peptides with a Cys residue at either the N or C terminus, attached via a flexible Gly-Gly linker (ACID-pLL^N and ACID-pLL^C, respectively), are mixed with an equimolar quantity of BASE-pLL (lacking a Cys residue) to complete formation of the heterotetramer. Disulfide-bonded ACID-pLL^N/ACID-pLL^N and ACID-pLL^C/ACID-pLL^C peptides are favored if the variant ACID-pLL helices are parallel with respect to each other in the heterotetramer. Conversely, disulfide-bonded ACID-pLL^N/ACID-pLL^C is favored if the variant ACID-pLL helices are antiparallel with respect to each other in the heterotetramer. The arrows point to the N terminus of the peptide. Analogous experiments were performed to determine the orientation of the BASE-pLL helices. (B) HPLC analysis of ACID-pLL orientation in the heterotetramer. The reduced ACID-pLL^N and ACID-pLL^C peptides oxidize to form disulfide-bonded species corresponding to both the parallel and antiparallel orientations of ACID-pLL helices with respect to each other, although the antiparallel orientation predominates. Disulfide-bonded ACID-pLL^N/ACID-pLL^C peptides rearrange to give the same species obtained from the reduced ACID-pLL^N and ACID-pLL^C peptides (unpublished results), indicating that the reaction mixture is at equilibrium. Experiments were performed in the presence of an equimolar amount of BASE-pLL. (C) HPLC analysis of BASE-pLL orientation in the heterotetramer. The reduced BASE-pLL^N and BASE-pLL^C peptides oxidize to form disulfide-bonded species corresponding to approximately equal fractions of parallel and antiparallel orientations of the BASE-pLL helices with respect to each other. An equimolar mixture of disulfide-bonded BASE-pLL^N/BASE-pLL^N and BASE-pLL^C/BASE-pLL^C rearranges to give the same species obtained from the reduced BASE-pLL^N and BASE-pLL^C peptides (unpublished results), indicating that the reaction mixture is at equilibrium. Experiments were performed in the presence of an equimolar amount of ACID-pLL. ACID-pLL^N and BASE-pLL^N have an additional N-terminal Trp residue to facilitate HPLC separation and so have larger extinction coefficients at 280 nm than ACID-pLL^C and BASE-pLL^C.

oligomerization orders [Hill et al., 1990; Lovejoy et al., 1993; Betz et al., 1995; see also Harbury et al. (1993)]. Such *de novo* designs often exclusively employ Leu residues to form the interhelical hydrophobic interface.

In contrast to other designed helical bundles, the ACID-p1/BASE-p1 heterodimer (O'Shea et al., 1993) has a unique, well-defined structure typical of native proteins, as shown collectively by the single (parallel) arrangement of helices, thermally induced and urea-induced sigmoidal unfolding transitions, lack of ANS binding, and hydrogen exchange rates for a subset of amide protons 10-fold slower than expected if exchange occurred by a global unfolding mechanism.

An explicit design feature of the ACID-p1/BASE-p1 heterodimer is a buried polar residue, Asn 14, in an otherwise hydrophobic interface (Figure 1). In the crystal structure of the GCN4 leucine zipper coiled coil, an analogous Asn forms a buried hydrogen bond to the corresponding Asn of the opposing helix (O'Shea et al., 1991). It was anticipated that the buried polar interaction involving Asn 14 would confer structural uniqueness to the ACID-p1/BASE-p1 heterodimer (O'Shea et al., 1993). Asn 14 has been replaced by Leu in the peptides ACID-pLL and BASE-pLL.

ACID-pLL and BASE-pLL form an extraordinarily stable heterotetramer that lacks structural uniqueness, as shown by the fast amide exchange rates, the weak binding of ANS, and the mixed orientations of helices. [The homotrimer Coil-Ser, which also has a predominantly Leu interface, also contains helices in both parallel and antiparallel orientations (Lovejoy et al., 1993).] Thus, the single buried polar interaction involving Asn 14 in the ACID-p1/BASE-p1 heterodimer introduces a native level of specificity for folding as a well-packed, two-stranded, parallel, coiled coil.

Interhelical packing constraints for different hydrophobic residues at positions **a** and **d** appear to influence strongly the oligomerization order of variant GCN4 leucine zippers that fold as two-, three-, or four-stranded coiled coils (Harbury et al., 1993, 1994). In the GCN4 leucine zipper, placing Leu residues at positions **a** and **d** results in a trimer. In contrast, the isolated ACID-pLL and BASE-pLL peptides fold as homodimers or homotetramers and, when mixed, form a heterotetramer. Other designed coiled coils with Leu at positions **a** and **d** form trimers (Betz et al., 1995) or tetramers (Zhu et al., 1993). Thus, packing interactions involving Leu residues at positions **a** and **d** are unable to impart specificity for a single oligomerization order to helical

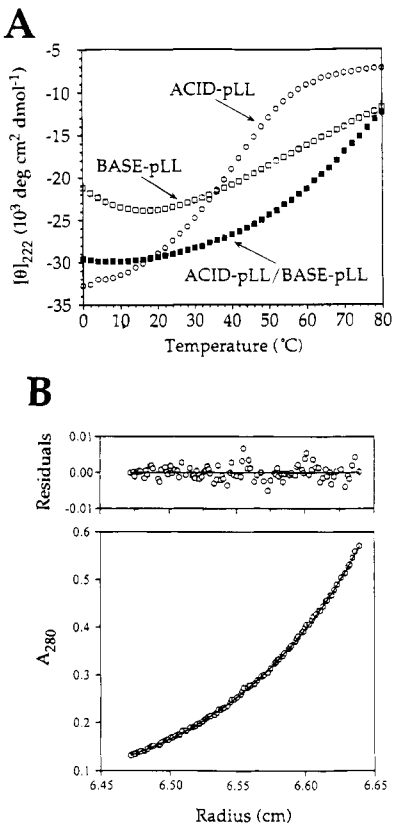

FIGURE 5: ACID-pLL and BASE-pLL form a heterotetramer under the conditions of the disulfide-exchange experiments (25 °C, 50 mM Tris-base, 150 mM NaCl, 2 M GdnHCl, pH 8.7). (A) CD experiments analogous to those of Figure 2B indicate that the ACID-pLL and BASE-pLL peptides associate preferentially under the redox conditions (10 μM total peptide concentration). (B) Sedimentation equilibrium indicates that the ACID-pLL and BASE-pLL peptides form a tetramer under the redox conditions (ACID-pLL/BASE-pLL at pH 8.7: observed molecular mass 14.4 kDa; expected for tetramer 14.2 kDa). The random distribution of residuals indicates that the data fit well to an ideal single-species model.

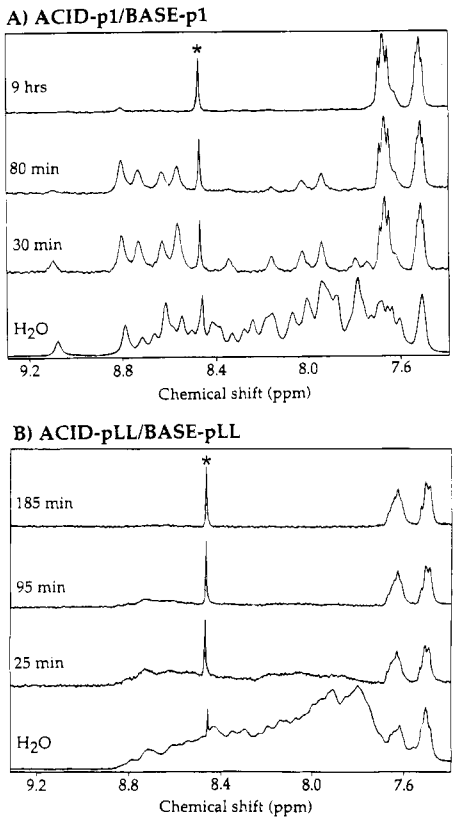

FIGURE 6: ^{1}H NMR amide hydrogen exchange studies of the ACID-p1/BASE-p1 heterodimer and the ACID-pLL/BASE-pLL heterotetramer (25 °C, pH 7.0, 1 mM total peptide concentration). The resonance indicated by an asterisk arises from a contaminant. (A) The slowest exchanging amide protons of the ACID-p1/BASE-p1 heterodimer have rates expected if exchange occurred via a global unfolding mechanism, suggesting the presence of a well-packed interior (see text). Under the conditions of the exchange experiment, the predicted protection factor ($1/f_u$) is 10^4. k_{int}/k_{ex} is 10^5 for the three most protected amides of the ACID-p1/BASE-p1 heterodimer. These results are in accord with previous studies at pH 4.7 (O'Shea et al., 1993). (B) The most protected amide protons of the ACID-pLL/BASE-pLL heterotetramer exchange 4 orders of magnitude more rapidly than expected for a global unfolding mechanism, suggesting a fluctuating structure. Under the conditions of the exchange experiment, the predicted protection factor ($1/f_u$) is 10^6. Since amide proton exchange is complete within 185 min, the upper limit of k_{ex}/k_{int} is 10^2. The NMR spectrum of the ACID-pLL/BASE-pLL heterotetramer is independent of concentration over the range 1 mM to 50 μM (where ACID-pLL/BASE-pLL is tetrameric by sedimentation equilibrium), indicating that the heterotetramer is not aggregating under the conditions of the NMR experiments.

bundles. The ACID-pLL and BASE-pLL peptides differ only at positions **e** and **g** and have different preferences for the parallel and antiparallel orientations. Although the structural basis for this difference between the ACID-pLL and BASE-pLL peptides is unclear, these results suggest that residues at positions **e** and **g** contribute to helix orientation in a coiled coil. Collectively, these results indicate that using only Leu residues at positions **a** and **d** should be avoided in the design of helical bundles and that residues at positions other than **a** or **d** influence both the oligomerization order and helix orientation of a coiled coil.

Our results, along with studies of the GCN4 leucine zipper (O'Shea et al., 1991, 1993; Harbury et al., 1993; Potekhin et al., 1994) and other designed coiled coils (Betz et al., 1995), suggest that a general method for introducing specificity for a parallel, two-stranded coiled coil is to include an Asn residue at position **a**. Moreover, the presence of a position **a** Asn provides a useful guide for predicting natural two-stranded coiled coils from amino acid sequences. The results also reinforce the notion (O'Shea et al., 1991) that conserved polar residues (typically Asn; Hu & Sauer, 1992; Hurst, 1994) at position **a** of leucine zippers promote formation of dimeric bZIP transcription factors.

Interior polar residues, such as Asn 14, presumably introduce structural uniqueness by destabilizing alternative conformations in which the buried polar side chain packs against hydrophobic residues and/or the hydrogen bonding potential of the polar side chain is unsatisfied (Chothia, 1976; O'Shea et al., 1991, 1993; Hendsch & Tidor, 1994; Betz et al., 1995).

Our results support a model for protein folding in which nonspecific hydrophobic interactions contribute stability but do not readily impart the high level of structural uniqueness typical of most native proteins (Dill, 1990; Handel et al., 1993). The complementary packing of different hydrophobic side chains or the formation of metal-binding sites can contribute some level of uniqueness to protein structure (Raleigh & DeGrado, 1992; Handel et al., 1993; Munson et al., 1994; Waldburger et al., 1995). In addition, we find that buried polar interactions, a common yet destabilizing feature of globular proteins, provide an elegant mechanism

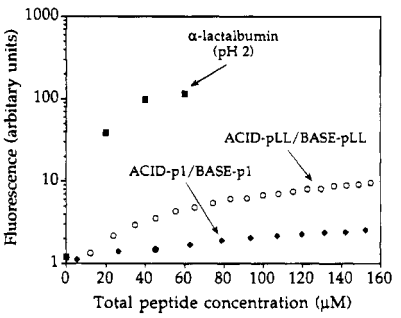

FIGURE 7: Fluorescence studies of ANS binding (PBS, 25 °C). The fluorescence intensity of ANS increases markedly (~10−100-fold), and the emission maximum shifts to ~470 nm upon binding to loosely packed proteins such as the molten globule form of α-lactalbumin. ANS binds to the ACID-pLL/BASE-pLL heterotetramer at pH 7.0 (the apparent K_d is 0.2 mM, and the ANS emission maximum is shifted to 470 nm), although the increase in ANS fluorescence is small compared to that of the molten globule form of α-lactalbumin (pH 2). In contrast, ANS does not bind substantially to the ACID-p1/BASE-p1 heterodimer at either pH 7.0 or pH 2.0 (unpublished results).

for introducing native-like levels of structural uniqueness to protein folding and design.

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