Complementation of Buried Lysine and Surface Polar Residues in a Designed Heterodimeric Coiled Coil†

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ABSTRACT: The coiled coil is an attractive target for protein design. The helices of coiled coils are characterized by a heptad repeat of residues denoted **a** to **g**. Residues at positions **a** and **d** form the interhelical interface and are usually hydrophobic. An established strategy to confer structural uniqueness to two-stranded coiled coils is the use of buried polar Asn residues at position **a**, which imparts dimerization and conformational specificity at the expense of stability. Here we show that polar interactions involving buried position-**a** Lys residues that can interact favorably only with surface **e′** or **g′** Glu residues also impart structural uniqueness to a designed heterodimeric coiled coil with the nativelike properties of sigmoidal thermal and urea-induced unfolding transitions, slow hydrogen exchange and lack of ANS binding. The position-**a** Lys residues do not, however, confer a single preference for helix orientation, likely reflecting the ability of Lys at position **a** to from favorable interactions with **g′** or **e′** Glu residues in the parallel and antiparallel orientations, respectively. The Lys-Glu polar interaction is less destabilizing than the Asn-Asn **a**→**a′** interaction, presumably reflecting a higher desolvation penalty associated with the completely buried polar position-**a** groups. Our results extend the range of approaches for two-stranded coiled-coil design and illustrate the role of complementing polar groups associated with buried and surface positions of proteins in protein folding and design.

Protein design provides a critical test of ideas regarding protein folding and stability, and holds promise for the rational creation of proteins with new properties. A popular target for protein design is the α-helical coiled coil (*1*−*4*). Naturally occurring coiled coils govern protein−protein interactions in a wide variety of biological processes (*5*, *6*), and over 5% of putative open reading frames in sequenced genomes are predicted to contain coiled-coil domains (*7*). Rules derived from coiled-coil design, therefore, have implications for predicting and understanding the properties of natural coiled coils as well as extending the repertoire of approaches to designing new sequences that fold and assemble in a predetermined manner.

The helices of the coiled coil are characterized by a heptad repeat of seven amino acids labeled **a**−**g** (*8*, *9*). Residues at positions **a** and **d** are usually hydrophobic, whereas residues at **e** and **g** positions are often charged (*8*, *9*). The hydrophobic interface between the helices of a coiled coil comprises the side chains of residues at positions **a** and **d**, and the methylene groups of the **e** and **g** residues (*10*−*14*). Polar side chains at positions **e** and **g** can also participate in intra- and interhelical electrostatic interactions (*10*−*14*).

Coiled-coil designs have utilized a variety of structural features such as hydrophobic packing at the **a**−**d** interface, interhelical **a**→**a′** buried polar interactions, and interhelical **g′**→**e** electrostatic interactions (*1*−*4*). The potential for exploiting interhelical interactions between normally buried position-**a** residues of one helix and normally solvent-exposed **e′** and **g′** side chain groups of the opposing helix has yet to be explored.

ACID-p1/BASE-p1 is a designed parallel coiled-coil heterodimer (*15*). ACID-p1 and BASE-p1 heterodimerize with high specificity, but the individual peptides do not form stable homodimers at neutral pH (*15*). The ACID-p1/BASE-p1 heterodimer exhibits sigmoidal thermal- and urea-induced unfolding transitions, slow hydrogen exchange, and a lack of ANS[1] binding (*15*, *16*). These nativelike properties suggest the presence of conformational specificity or structural uniqueness, i.e., a well-defined, single fold (or family of very closely related folds) with a well-packed hydrophobic interior (*15*, *16*).

An explicit design feature of ACID-p1/BASE-p1 is a buried polar interaction between two position-**a** Asn residues (*15*). Substitution of the position-**a** Asn with Leu to form the peptides ACID-pLL and BASE-pLL results in the

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[1] Abbreviations: $\Delta G_u°$, free energy of unfolding; $[\theta]$, molar ellipticity; $[\theta]_{222}$, molar ellipticity at 222 nm; ANS, 1-anilinonaphthalene-8-sulfonic acid; CD, circular dichroism; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; f_u, fraction unfolded; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline (10 mM sodium phosphate and 150 mM NaCl); T_m, midpoint of thermal denaturation.

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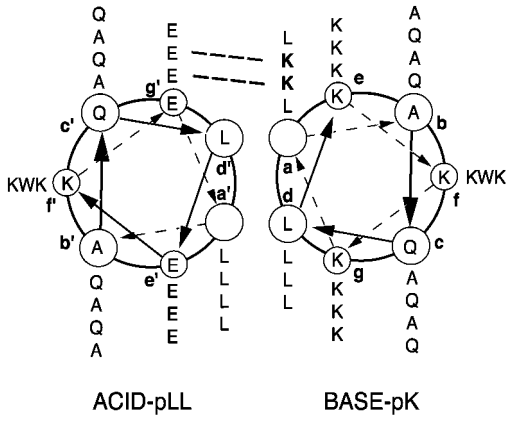

FIGURE 1: Helical-wheel representation of the ACID-pLL/BASE-pK heterodimer shown as a parallel coiled coil. In the parallel ACID-pLL/BASE-pK heterodimer, the ϵ-amino group of the position-**a** Lys can only form favorable polar interactions with position-**g′** Glu residues of ACID-pLL, shown using dashed lines. In the antiparallel orientation, the ϵ-amino group of the position-**a** Lys can only form favorable polar interactions with position-**e′** Glu residues of ACID-pLL (not shown). The position-**d** Leu residues of ACID-pLL are omitted for clarity. For both peptides, the N-termini are acetylated, and the C-termini are amidated.

formation of a very stable heterotetramer (*16*). The ACID-pLL/BASE-pLL heterotetramer does not fold in a unique conformation, since the helices lack a single orientation and the heterotetramer exhibits the molten-globule properties of low protection from hydrogen exchange and ANS binding (*16*). The Asn residues presumably impart structural uniqueness by destabilizing conformations that do not satisfy the hydrogen-bonding potential of the Asn side chains (*16*). The conformationally plastic peptides ACID-pLL and BASE-pLL provide a platform to test the ability of different interactions to confer structural uniqueness to protein structure.

In a parallel two-stranded coiled coil, Lys at position **a** can form polar interactions with position-**g′** residues of the preceding heptad of the opposing helix (*17*). In an antiparallel coiled coil, polar residues at position **a** can form polar interactions with position-**e′** residues of the opposing helix (*10*, *14*). Two position-**a** Lys residues were placed into the context of BASE-pLL to make BASE-pK (Figure 1), with the expectation that interhelical polar interactions between the position-**a** Lys residues of BASE-pK and **e′** or **g′** Glu residues of ACID-pLL could impart structural uniqueness and dimerization specificity. In a dimeric assembly, the only residues able to make a favorable polar interaction with the position-**a** Lys ϵ-amino groups of BASE-pK are the **e′** or **g′** Glu residues of ACID-pLL in the antiparallel or parallel orientations, respectively (Figure 1).

We find that the position-**a** Lys residues do indeed impart a significant level of structural uniqueness: ACID-pLL and BASE-pK associate to form a heterodimer with the properties expected of a native globular protein. There is not, however, a significant preference for a single orientation of helices (parallel or antiparallel) in the ACID-pLL/BASE-pK heterodimer. Our results enlarge the range of combinatorial interactions that can be utilized in coiled-coil prediction, design, and assembly.

EXPERIMENTAL PROCEDURES

The peptides ACID-p1, BASE-p1, ACID-pLL (corresponding to ACID-p1 with Asn 14 substituted for Leu: Figure 1), ACID-pLL^N (corresponding to ACID-pLL with an N-terminal Ac-Cys-Gly-Gly), and ACID-pLL^C (corresponding to ACID-pLL with a C-terminal Gly-Gly-Cys-NH_2) were kind gifts of Peter S. Kim (*15*, *16*). BASE-pK (Figure 1) was synthesized with Fmoc chemistry by the Macromolecular Resources Facility at Colorado State University. BASE-pK^N, corresponding to BASE-pK with an N-terminal Ac-Cys-Gly-Gly, was synthesized on MBHA (4-methylbenzhydrylamine hydrochloride salt) resin (100−200 mesh) using manual Boc chemistry (*18*). The side chains of Cys, Gln, Lys, and Trp were protected during Boc synthesis with MeBzl, Xan, 2-Cl-Z, and CHO, respectively. Peptides were purified by reversed-phase C_{18} HPLC using linear water/acetonitrile gradients containing 0.1% (v/v) trifluoroacetic acid. The identity of each peptide was confirmed with electrospray mass spectrometry, and in all cases, the expected and observed masses agreed to within 1 Da.

Concentrations of peptide stock solutions were determined by Trp absorbance in 6 M GuHCl, 10 mM sodium phosphate, and 50 mM NaCl, pH 6.5, using an extinction coefficient at 280 nm of 5690 cm^{-1} M^{-1} (*19*).

CD spectra were acquired with Aviv 202 or Jasco J720 CD spectrometers. Samples contained 10 μM peptide in PBS (10 mM sodium phosphate and 150 mM NaCl), pH 7.0.

Thermal stability was monitored from the change in the CD signal at 222 nm with temperature with an Aviv 202 CD spectrometer. Samples contained 10 μM peptide in PBS, pH 7.0. The temperature was increased in steps of 2 °C with an equilibration time of 60 s. The CD signal at 222 nm was averaged over 60 s. The T_m was determined from the maxima of the first derivative of $[\theta]_{222}$ with respect to T^{-1} (*20*).

The free energy of unfolding at 25 °C was determined from the change in the CD signal at 222 nm with urea concentration with an Aviv 202 CD spectrometer. Samples contained 10 μM ACID-pLL/BASE-pK in PBS, pH 7.0. Urea concentration was increased in steps of 0.4 M with a 600 s equilibration time, and the CD signal at 222 nm was averaged over 60 s. Data were fit to an unfolded monomer−folded dimer equilibrium to obtain the equilibrium constant for unfolding at each urea concentration, K_u, given by: $2 \cdot c \cdot f_u^2/(1 - f_u)$, where c is the total peptide concentration and f_u is the fraction unfolded. The change in $\Delta G_u°$, given by $-RT \ln K_u$, with urea concentration ([urea]) in the transition region was fit to $\Delta G_u°([\text{urea}]) = \Delta G_u°(\text{PBS}) + m[\text{urea}]$ to obtain $\Delta G_u°(\text{PBS})$ and m (*21*). Urea concentration was determined with refractometry (*22*).

Sedimentation equilibrium experiments were performed with a Beckman XL-I analytical ultracentrifuge. Samples were dialyzed for at least 12 h against the reference buffer (PBS, pH 7.0). Data were collected at 25 °C at rotor speeds of 40 and 48 krpm using two-sector and six-sector centerpieces. For BASE-pK, eight data sets were collected at 280 nm with loading concentrations ranging from 20 to 80 μM. For ACID-pLL/BASE-pK, 6 and 14 data sets were collected at 230 and 280 nm, respectively, with loading concentrations ranging from 10 to 100 μM. A solvent density of 1.004 g mL^{-1} and calculated partial specific volumes of 0.783 mL g^{-1} (BASE-pK) and 0.762 mL g^{-1} (ACID-pLL/BASE-pK)

were used (*23*). Data in the approximate concentration range 6−90 μM were fit using ORIGIN (Beckman Instruments) to an ideal, single-species model, with the validity of the model evaluated by the distribution of residuals. Masses are reported as the mean mass of the individual fits ± the standard error.

Helix orientation was probed by the concentration dependence of stability of disulfide-bonded peptides (*24*, *25*). The helices of ACID-pLL and BASE-pK were constrained to be parallel by forming the disulfide between ACID-pLL^N and BASE-pK^N, and were constrained to be antiparallel by forming the disulfide between ACID-pLL^C and BASE-pK^N. The disulfide-bonded peptides were prepared by incubating the HPLC-purified reduced peptides in 6 M GuHCl, pH 8, at room temperature overnight. The desired disulfide-bonded heterodimer (i.e., ACID-pLL^N/BASE-pK^N or ACID-pLL^C/BASE-pK^N) was separated from the reduced peptides or the unwanted disulfide-bonded homodimers by C_{18} HPLC. The identities of the disulfide-bonded products were confirmed with electrospray mass spectrometry, and the expected and observed masses agreed to within 1 Da. The T_m values of the disulfide-bonded peptides were determined with CD spectroscopy as described above, except samples were prepared in 10 mM sodium phosphate, 150 mM NaCl, and 3 M GuHCl, pH 7.0, containing peptide at total concentrations of 5, 10, 20, and 30 μM. The GuHCl concentration was determined with refractometry (*22*).

Hydrogen exchange experiments were performed with Varian Unity Inova NMR spectrometer operating at 500.13 MHz for ^{1}H. Samples contained 0.5 mM ACID-pLL/BASE-pK in PBS, pH 7.0, and were internally referenced to DSS at zero ppm (*26*). One-dimensional ^{1}H spectra were acquired at 25 °C with a spectral width of 6000.1 Hz. Data sets consisted of 2048 transients defined by 8192 complex points. Data were resolution enhanced with a shifted Gaussian function and zero filled once prior to Fourier transformation, and the baseline was corrected, using VNMR 6.1b software (Varian). Hydrogen exchange studies were initiated by dissolving lyophilized samples in D_2O. The pH of the solution was checked after the experiment and found to be 7.01, uncorrected for the isotope effect. The observed hydrogen exchange rate (k_{ex}) was obtained from fitting the change in amide resonance intensity (I) with time (t) to: $I = I_0 \exp(-k_{ex}t) + I_\infty$. The intrinsic exchange rate (k_{int}) was taken to be 31 s^{-1} (*16*), corresponding to 3 times the rate of amide exchange for poly-DL-alanine at pH 7.0 and 25 °C (*27*). The observed protection factor was calculated as k_{ex}/k_{int}. The expected protection factor was calculated from $1/f_u$, which assumes a global unfolding mechanism of hydrogen exchange for a stable protein ($f_u \ll 1$) (*28*). f_u was calculated from: $K_u = 2 \cdot c \cdot f_u^2/(1 - f_u)$, where K_u is the equilibrium constant for unfolding determined by urea denaturation as described above and c is the total peptide concentration.

Fluorescence studies of ANS binding were performed at 25 °C with an Aviv ATF105 fluorometer. Emission spectra were collected using an excitation wavelength of 355 nm and an emission wavelength of 400−600 nm. Samples contained 5 μM ANS (Molecular Probes) and 0−100 μM bovine α-lactalbumin in PBS, pH 2, or 0−100 μM ACID-pLL/BASE-pK in PBS, pH 7.0. ANS concentration was determined for a stock solution in methanol by the absorbance at 372 nm using an extinction coefficient of 7.8 × 10^3 cm^{-1} M^{-1}.

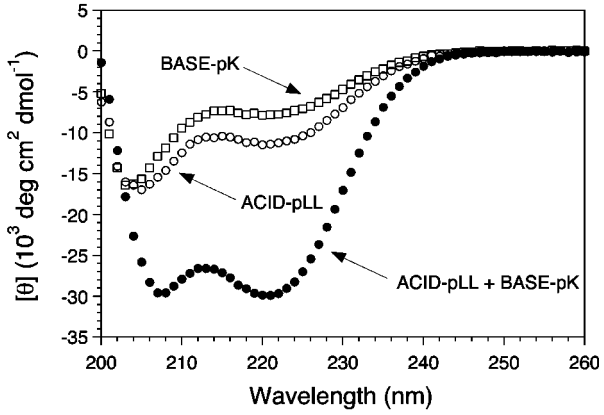

FIGURE 2: CD spectra of ACID-pLL, BASE-pK, and ACID-pLL/BASE-pK (PBS, pH 7.0, 25 °C, total peptide concentration 10 μM). The minima at 208 and 222 nm indicate that the peptides form helical structures. The CD spectrum of an equimolar mixture of ACID-pLL and BASE-pK is not the average of the spectra of the isolated ACID-pLL and BASE-pK peptides, indicating that the peptides associate to form a heterospecies.

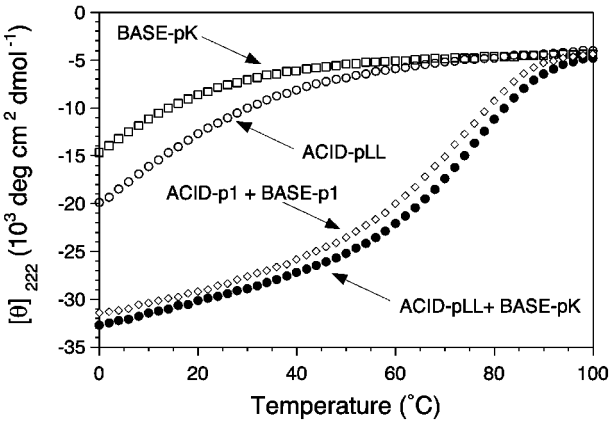

FIGURE 3: Thermal stability of ACID-pLL, BASE-pK, and ACID-pLL/BASE-pK monitored by the temperature dependence of $[\theta]_{222}$ (PBS, pH 7.0, total peptide concentration 10 μM). Thermal melts were reversible (the forward and reverse melts were superimposable, with over 98% of the starting signal regained on cooling).

RESULTS

Properties of the Isolated ACID-pLL and BASE-pK Peptides. The CD spectrum of ACID-pLL indicates that the peptide forms a partially helical structure (Figure 2), in accord with previous results (*16*). The temperature dependence of $[\theta]_{222}$ indicates that the helical structure is marginally stable, in accord with previous results (*16*), with an apparent T_m of approximately 0 °C (Figure 3). CD indicates that BASE-pK also forms a marginally stable helix with an apparent T_m of approximately 0 °C (Figures 2 and 3). Since BASE-pLL forms a stable, helical homodimer with a T_m of 64 °C under the same conditions as used here (*16*), the substitution of Leu with Lys at position **a** of BASE-pK is destabilizing.

Sedimentation equilibrium indicates that BASE-pK is predominantly monomeric. The expected mass for the monomer is 3.585 kDa, and the apparent mass obtained from a single-species fit is 4.70 ± 0.4 Da (data not shown).

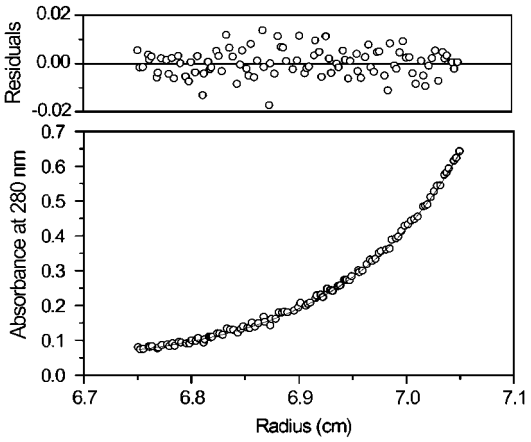

FIGURE 4: Sedimentation equilibrium indicates that ACID-pLL/BASE-pK is dimeric (PBS, pH 7.0, 25 °C). The observed molecular mass is 7.60 ± 0.4 Da (expected for the heterodimer: 7.148 kDa). The random distribution of residuals indicates that a single-species model accounts for the data. Systematic changes in apparent molecular mass with total peptide concentration or rotor speed are not observed.

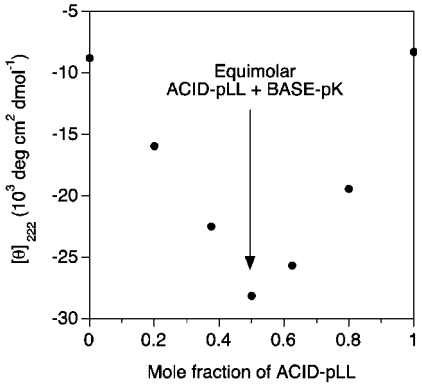

FIGURE 5: Stoichiometry of ACID-pLL/BASE-pK assembly. The helix content of the ACID-pLL + BASE-pK solution is maximal when the peptides are mixed in equimolar amounts, indicating that the peptides associate with a 1:1 stoichiometry (PBS, pH 7.0, 25 °C, total peptide concentration 10 μM).

Previous studies show that ACID-pLL exists in a monomer−dimer−tetramer equilibrium that favors the monomer at low micromolar concentrations (*16*).

Heterodimerization of ACID-pLL and BASE-pK. On mixing, changes in the CD spectrum show that ACID-pLL and BASE-pK associate to form a new helical species with a helix content significantly greater than the isolated peptides (Figure 2). Sedimentation equilibrium indicates that the ACID-pLL and BASE-pK peptides form a dimer (Figure 4). In contrast, the isolated ACID-pLL and BASE-pK peptides are predominantly monomeric (see above). The helical content of the heterodimer is maximal when ACID-pLL and BASE-pK are mixed in equimolar amounts, indicating that the ACID-pLL and BASE-pK peptides associate with a 1:1 stoichiometry (Figure 5). The ACID-pLL/BASE-pK heterodimer is dramatically more stable than either of the two isolated peptides (Table 1), with a T_m of 74 °C (Figure 3) and a free energy of unfolding ($\Delta G_u°$) measured by urea denaturation of 11.7 kcal mol^{-1} (Figure 6). The ACID-pLL/BASE-pK heterodimer is more stable than the ACID-p1/BASE-p1 heterodimer, for which the T_m is 72 °C (Figure 3) and the $\Delta G_u°$ is 10.1 kcal mol^{-1} (*15*). ACID-pLL and BASE-pK therefore assemble to form a stable, helical heterodimer.

Table 1: Summary of Thermodynamic Parameters for the ACID/BASE Family of Peptides

peptide	buffer	T_m[a] (°C)	$\Delta G_u°$ (kcal mol^{-1})
ACID-p1/BASE-p1	PBS	72	10.1[b]
ACID-pLL/BASE-pK	PBS	74	11.7
ACID-pLL	PBS	∼0	
BASE-pLL	PBS	∼0	
ACID-pLL/BASE-pLL	PBS	>100[c]	
BASE-pK	PBS	∼0	
ACID-pLL^N/BASE-pK^N	3 M GuHCl, PBS	58	
ACID-pLL^C/BASE-pK^N	3 M GuHCl, PBS	58	

[a] T_m values at a total peptide concentration of 10 μM. [b] O'Shea et al. (*15*). [c] Lumb and Kim (*16*).

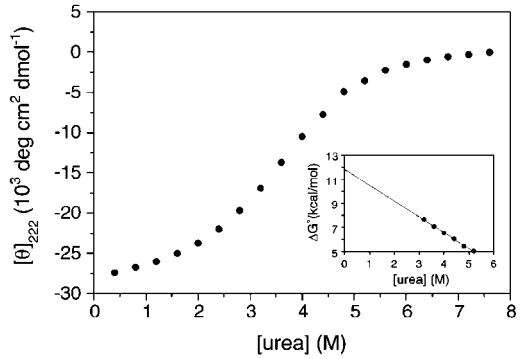

FIGURE 6: Stability of the ACID-pLL/BASE-pK heterodimer (PBS, pH 7.0, 25 °C, total peptide concentration 10 μM). The change in $[\theta]_{222}$ with urea concentration fit a monomer−dimer equilibrium (inset) to yield an apparent $\Delta G_u°$ of 11.7 kcal mol^{-1} (m = 1.3 kcal mol^{-1} M^{-1}). The standard error in $\Delta G_u°$ calculated from independent measurements is 0.2 kcal mol^{-1}.

Helix Orientation of the ACID-pLL/BASE-pK Heterodimer. Helix orientation in coiled coils can be determined from the concentration dependence of stability of disulfide-bonded peptides (*24*, *25*). The stability and helix content of a disulfide-bonded dimer are independent of concentration when the helices are joined in the preferred orientation. Conversely, stability and helix formation increase with concentration when helices are covalently cross-linked in the unfavorable orientation, arising from the intermolecular association of higher-order assemblies with the helices in the preferred orientation (*24*, *25*). For example, in PBS containing 2.25 M GuHCl at pH 7.0, $[\theta]_{222}$ at 0 °C of the ACID-p1 and BASE-p1 peptides when disulfide-bonded in the unfavorable (antiparallel) orientation changes from −11 100 to −25 300 deg cm^2 dmol^{-1} as the concentration is increased from 9 to 25 μM (*15*).

ACID-pLL and BASE-pK cross-linked in both the parallel orientation and antiparallel orientations have T_m values that are independent of concentration. The T_m values of the parallel disulfide-bonded ACID-pLL^N/BASE-pK^N and antiparallel disulfide-bonded ACID-pLL^C/BASE-pK^N exceed 100 °C in PBS, pH 7 (data not shown). In PBS containing 3 M GuHCl, pH 7.0, the T_m is 58 ± 2 °C at total peptide concentrations of 5, 10, 20, and 30 μM for both ACID-pLL^N/BASE-pK^N and ACID-pLL^C/BASE-pK^N (Table 1). In PBS, pH 7.0 (containing no denaturant), the helix content of ACID-pLL^N/BASE-pK^N does not vary systematically over the concentration range 5−30 μM, with $[\theta]_{222}$ values at 5 and 25 °C of (26.7 ± 0.4) × 10^3 and (24.5 ± 0.5) × 10^3 deg cm^2 dmol^{-1}, respectively. $[\theta]_{222}$ for ACID-pLL^C/BASE-pK^N

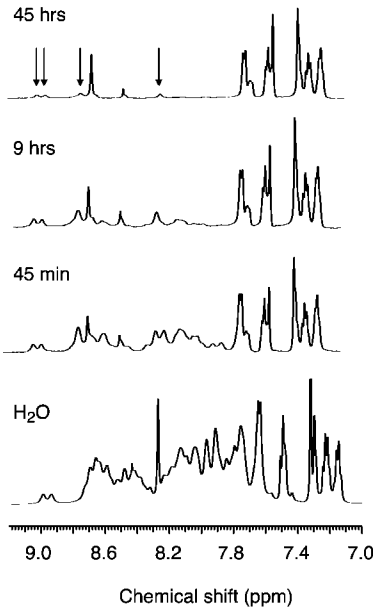

FIGURE 7: ^{1}H amide hydrogen exchange studies of the ACID-pLL/BASE-pK heterodimer (PBS, pH 7.0, 25 °C, 300 μM total peptide concentration). One-dimensional spectra are shown of ACID-pLL/BASE-pK in 90% H_2O/10% D_2O and at various time points after dissolution in D_2O. Fits of the change in amide intensity with time of the four most slowly exchanging amide resonances (indicated with arrows) yield protection factors of 10^6, which exceeds the predicted protection factor ($1/f_u$) of 10^3. This observation suggests the presence of a well-packed interior.

is also independent of concentration, with $[\theta]_{222}$ values at 5 and 25 °C of (26.9 ± 0.2) × 10^3 and (25.0 ± 0.2) × 10^3 deg cm^2 dmol^{-1}, respectively. The helices of the ACID-pLL/BASE-pK heterodimer, therefore, do not exhibit a marked preference for a single orientation.

Nativelike Properties of the ACID-pLL/BASE-pK Heterodimer. Native, globular proteins exhibit sigmoidal unfolding transitions, do not bind ANS, and have a subset of amide protons with hydrogen exchange protection factors comparable to those expected if exchange occurred only from the globally unfolded state. In contrast, molten globules and designed proteins with ill-defined or loosely packed structures exhibit linear thermal unfolding transitions, ANS binding, and low hydrogen exchange protection factors (*29*−*31*).

The predicted protection factor when hydrogen exchange occurs only from a globally unfolded protein is $1/f_u$ (*28*). The slowest-exchanging amide protons of native globular proteins often have protection factors comparable to or greater than those expected if exchange occurred from the globally unfolded state (*16*, *28*). This empirical observation forms the basis for using hydrogen exchange as a probe of nativelike, globular structure.

The slowest-exchanging amide protons of ACID-pLL/BASE-pK have protection factors of 10^6 (Figure 7), which is comparable to the protection factors observed in well-packed proteins and higher than the predicted value of 10^3 if exchange occurred from the globally unfolded state. A higher than predicted protection factor is seen for natural proteins (*16*, *28*) and for other coiled coils based on the ACID/BASE parent sequence with nativelike properties (*15*, *16*, *32*, *33*). For the ACID/BASE family of peptides with slowly exchanging protons, the higher than predicted protection factor may reflect residual structure in the unfolded state,

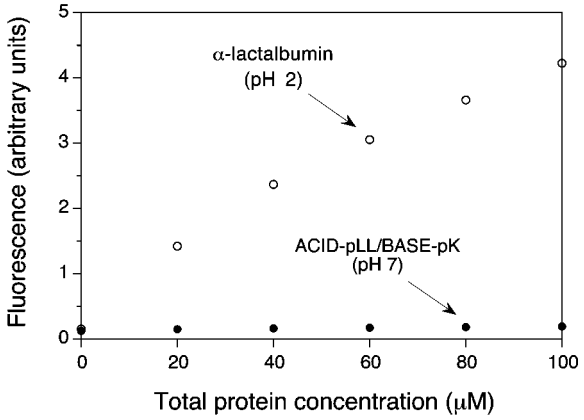

FIGURE 8: Fluorescence studies of ANS binding (PBS, 25 °C). The pH 2 α-lactalbumin molten globule induces a significant increase in ANS fluorescence intensity and a shift in the ANS emission maximum from 484 to 470 nm, indicating that the α-lactalbumin molten globule binds ANS in accord with previous results (*36*). In contrast, the ACID-pLL/BASE-pLL heterodimer at pH 7.0 does not induce significant changes in ANS fluorescence, indicating that the heterodimer does not bind appreciably to ANS.

differences in stability in D_2O and H_2O, or deviations from a strict two-state unfolding mechanism (*28*, *33*). The presence of slowly exchanging amide protons in the ACID-pLL/BASE-pK heterodimer suggests the presence of a well-packed hydrophobic core typical of natural, globular proteins.

ANS binding to proteins is accompanied by a significant increase in ANS emission intensity and a decrease in emission wavelength from 500 nm to approximately 470 nm (*34*). Molten globules and designed proteins that lack structural uniqueness bind ANS, presumably because these proteins are loosely packed (*35*−*37*). Such large changes in ANS fluorescence are induced here by the pH 2 molten-globule form of α-lactalbumin, in accord with previous results (*36*). In contrast, minimal changes in ANS fluorescence are induced by the ACID-pLL/BASE-pK heterodimer, indicating that ANS does not bind the heterodimer to an appreciable extent (Figure 8).

The heterodimer lacks structural uniqueness in the sense that there is not a single, preferred orientation of helices. However, the sigmoidal thermal- and urea-unfolding transitions (Figures 2 and 6), slow hydrogen exchange (Figure 7), and lack of ANS binding (Figure 8) of the ACID-pLL/BASE-pK heterodimer are consistent with the properties expected of a native protein with a well-defined fold, as opposed to a fluctuating or poorly packed structure reminiscent of the molten globule.

DISCUSSION

Successful protein design requires a balance of hydrophobic interactions for stability and directional interactions to confer structural uniqueness. Approaches to imparting structural uniqueness include considerations of core packing and binary patterns of hydrophobic and polar residues (*38*, *39*). Another approach is the use of buried polar interactions, with the intent of destabilizing conformations that do not satisfy the hydrogen-bonding or salt-bridging potential of polar groups (*15*, *16*, *32*, *40*−*42*).

Asn and Lys at position **a** are the most common buried polar residues in two-stranded natural coiled coils (*43*−*45*). The role of position-**a** Asn residues has often been studied.

Asn at position **a** strongly promotes dimer formation in both natural and designed coiled coils (*11*, *12*, *16*, *40*, *46*−*52*) and imparts structural uniqueness in the designed heterodimeric coiled-coil ACID-p1/BASE-p1 (*16*).

Position-**a** Lys residues have received less attention (*49*, *51*, *53*). Lys at position **a** can form an *intra*helical electrostatic interaction with an **e**-position residue of the preceding heptad, as seen in the crystal structure of a parallel GCN4 variant dimer with an **a**-position Lys (*49*). In this case, the hydrophobic side chain of Lys forms part of the hydrophobic interface of the coiled coil, and the polar ϵ-amino group forms a polar interaction with the **e**-position polar group. In the context of ACID-pLL/BASE-pK, juxtaposition of the position-**a** and -**e** Lys residues of BASE-pK is expected to be destabilizing and unfavorable for coiled-coil formation.

Lys at position **a** can form an *inter*helical **g′**→**a** polar interaction with a **g′**-position polar residue of the preceding heptad of the opposing helix in a parallel dimer, as seen in the crystal structures of the Fos-Jun parallel coiled coil (*17*). In an antiparallel dimer, a polar residue such as Lys at position **a** can form an *inter*helical **e′**→**a** polar interaction with an **e′**-position polar residue of the preceding heptad of the opposing helix, as seen in crystal structures of the antiparallel coiled coils of seryl-tRNA synthetase and GreA (*10*, *14*). Interhelical interactions involving Lys at position **a** paired with an appropriate partner at position **e′** or **g′** may therefore offer an alternative route to position-**a** Asn residues for imparting fold specificity in designed coiled coils. Polar **g′**→**a** interactions involve partially buried position-**a** Lys residues and solvent-accessible **e′** or **g′** side chains on the surface of the coiled-coil interface, and so differ from buried **a**−**a′** interactions.

In the context of the ACID/BASE system, Lys at position **a** of BASE-pK has the potential to form an interhelical polar interaction with a position-**g′** Glu of the preceding heptad of the opposing helix of ACID-pLL in the parallel heterodimer (Figure 1), and with an **e′**-position Glu in the antiparallel heterodimer. These are the only stabilizing interactions available to the position-**a** Lys residues of BASE-pK, since formation of intrahelical electrostatic interactions between Lys residues at positions **a** and **e** is expected to be destabilizing. We find that incorporation of the position-**a** Lys residues in BASE-pK confers both dimerization specificity and a native level of core packing, but does not impart a significant preference for a particular orientation of helices to the ACID-pLL/BASE-pK heterodimer.

Various approaches have been used to confer specificity for the antiparallel and parallel orientations of helices, including pairing of position-**a** Asn residues and complementary **g′**−**e** electrostatic interactions (*4*). The position-**a** Lys residues of BASE-pK can conceivably form both interhelical **g′**→**a** and **e′**→**a** interactions in the parallel and antiparallel orientations, respectively (see above), and so the lack of a significant preference for a single helix orientation is perhaps not surprising. Imparting specificity for a single orientation might be attained by placing residues at **e′** or **g′** that form unfavorable interactions with the position-**a** Lys in the unwanted, but not in the desired, orientation.

Buried polar residues typically destabilize coiled coils relative to substitution with hydrophobic residues (*12*, *16*, *40*, *46*−*49*, *51*, *52*, *54*−*57*), which reflects the energetic cost of desolvating a buried polar group (*58*). This is seen here also; the ACID-pLL/BASE-pK heterodimer is less stable than the ACID-pLL/BASE-pLL heterotetramer, and BASE-pK is destabilized relative to BASE-pLL (Table 1). Interestingly, the ACID-pLL/BASE-pK heterodimer is more stable than the ACID-p1/BASE-p1 heterodimer (Table 1). The position-**a** Lys residues therefore impart dimerization specificity and nativelike levels of structural uniqueness at the expense of stability, compared to substitution with the hydrophobic residue Leu, but with a lower energetic penalty than incurred with two buried position-**a** Asn residues. This may reflect a smaller desolvation penalty for the surface-exposed Lys and Glu side chains than for the completely buried Asn side chains.

Buried polar interactions occur frequently in natural proteins and are expected to destabilize conformations in which the buried polar groups are not involved in hydrogen bonds or salt brides (*59*, *60*). Buried polar residues have been found to impart structural uniqueness in natural and designed coiled coils (*12*, *16*, *32*, *49*), designed helical bundles (*38*), and the globular protein thioredoxin (*61*). These findings, together with the results reported here, support the notion that directional polar interactions can contribute in a defining way to protein folding and structural uniqueness.

In conclusion, complementary interactions between buried polar groups, which lack a buried polar interaction partner, and surface polar groups can impart native levels of structural uniqueness to protein folding and design with a smaller energetic penalty than incurred through the use of a completely buried polar interaction. The application of buried surface electrostatic interactions provides an alternative route to the established role of buried polar interactions for introducing organization and specificity to protein design.

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