Design of Heterotetrameric Coiled Coils: Evidence for Increased Stabilization by Glu^--Lys^+ Ion Pair Interactions

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ABSTRACT: Electrostatic interactions between charged amino acids often affect heterospecificity in coiled coils as evidenced by the interaction between the oncoproteins, fos and jun. Such interactions have been successfully exploited in the design of heteromeric coiled coils in a number of laboratories. It has been suggested that heterospecificity in these dimeric coiled-coil systems is driven not by specific electrostatic interactions in the heterodimers but rather by electrostatic repulsion acting to destabilize the homodimer state relative to the heterodimer state. We show that it is possible to design ion pair interactions that directly stabilize the heterotetrameric coiled-coil state. Synthetic peptides were used whose sequences are based on the C-terminal tetramerization domain of Lac repressor, as a model system for four-chain coiled coils (Fairman et al., 1995). These Lac-based peptides, containing either glutamic acid (Lac21E) or lysine (Lac21K) at all b and c heptad positions, only weakly self-associate but, when mixed, afford a highly stable heterotetramer. This study represents the first experimental evidence for the importance of the b and c heptad positions to the stability of coiled coils. Finally, pH dependence and NaCl dependence studies show that heterotetramer stability is driven by ion pair interactions between glutamate and lysine; these interactions contribute about 0.6 kcal/mol of stabilizing free energy for each potential glutamate−lysine pair.

Coiled coils are α-helical protein structural motifs which act as multimerization modules. Intrinsic flexibility of helix-pairing interactions provides the opportunity for this motif to adopt multiple oligomerization states and to form heterospecific interactions, often through only a few changes in the polypeptide sequence. For example, mutations of the core residues of GCN4 (a and d heptad positions; see Figure 1) allow this coiled coil to form dimers, trimers, or tetramers (Harbury et al., 1993). The amino acid sequences of coiled coils are described frequently in units of heptad repeats, as there are exactly seven residues per two turns of α-helix. By convention, the seven positions within a heptad are denoted a−g (Figure 1).

Electrostatic effects can mediate oligomerization states (Alberti et al., 1993; Krylov et al., 1994) and can also define heterospecificity of interchain interactions. Electrostatic interactions are largely responsible for the pairing specificity between the coiled-coil domains from the oncoproteins fos and jun (O'Shea et al., 1992). Several recent X-ray crystal structures of coiled coils have helped us to understand the atomic details of the contribution of such electrostatic interactions to the structure of coiled coils (O'Shea et al., 1991; Harbury et al., 1993; Glover & Harrison, 1995). A number of laboratories have used this information to exploit electrostatic interactions in the design of heterodimeric coiled coils (O'Shea et al., 1993; Graddis et al., 1993; Krylov et al., 1994; Zhou et al., 1994).

To explore issues of specificity for coiled coils that form higher order oligomers, peptides based on the tetramerization domain of Lac repressor were used as a model system (Fairman et al., 1995). We had demonstrated previously that synthetic peptides derived from the C-terminal 21 amino acids of Lac repressor form four-chain coiled coils with

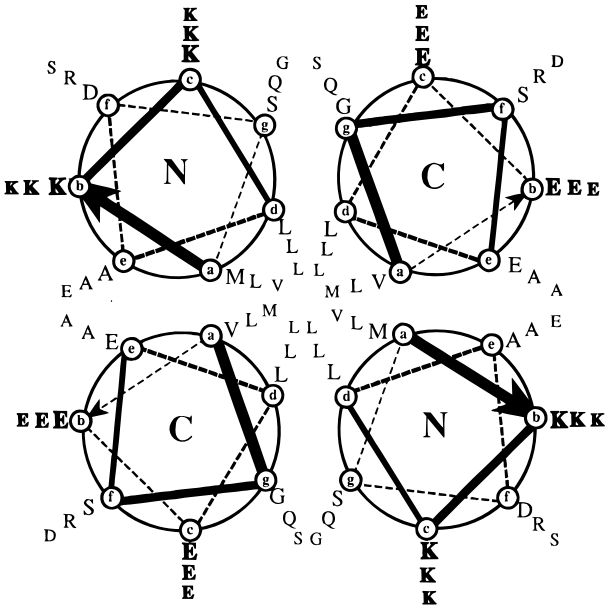

FIGURE 1: Helical wheel diagram showing E−K interactions at b and c positions for the Lac21E and Lac21K peptide sequences. The amino acid sequences for the peptides are

heptad position	abcdefg abcdefg abcdefg abcdefg
Lac21E:	Ac-MEELADS LEELARQ VEELESA-$CONH_2$
Lac21K:	Ac-MKKLADS LKKLARQ VKKLESA-$CONH_2$
Lac28E:	Ac-MEELADS LEELARQ LEELARQ VEELESA-$CONH_2$
Lac28K:	Ac-MKKLADS LKKLARQ LKKLARQ VKKLESA-$CONH_2$

The parent peptide sequence is

Lac21:	Ac-MKQLADS LMQLARQ VSRLESA-$CONH_2$

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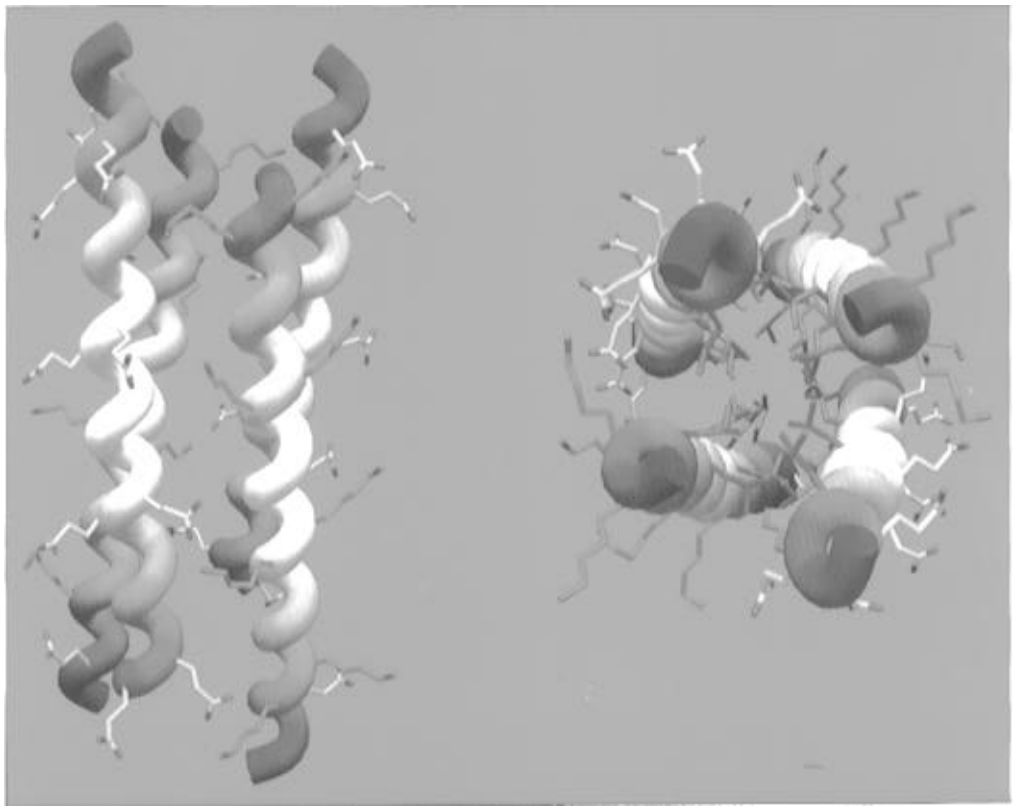

FIGURE 2: (A, left) Longitudinal view of an ICM-generated model of the antiparallel Lac28E/K heterotetramer. α-Helical backbones, shown as solid ribbons, are color-coded from the N-terminus to the C-terminus, blue to red. Lysine (green, blue) and glutamate (yellow, red) side chains are also shown. (B, right) View of an ICM-generated model of the antiparallel Lac28E/K heterotetramer, approximately down the interhelical axis. α-Helical backbones, shown as solid ribbons, are color-coded from the N-terminus to the C-terminus, blue to red. Lysine (green, blue), glutamate (yellow, red), and leucine (magenta, at the helical interface) side chains are also shown. Computer graphics generated with the program of GRASP of A. Nicholls (1991).

evidence for an antiparallel arrangement of the chains. The recent X-ray crystal structure of the Lac repressor core domain confirmed this model for the tetramerization domain (Friedman et al., 1995). Changes at e and g positions were avoided as they can affect the assembly of coiled coils (Krylov et al., 1994). Instead, on the basis of modeling of the Lac tetramers, charged residues were considered at b and c positions, two positions whose effects on coiled-coil formation have been largely unexplored (Figures 1 and 2). Synthetic peptides were studied containing either glutamic acid or lysine at both b and c positions in the background of the Lac sequence to determine oligomerization state and heterospecificity.

MATERIALS AND METHODS

Models of both parallel and antiparallel four-heptad, α-helical homotetramers derived from the Lac tetramerization domain amino acid sequence, were described by Fairman et al. (1995), on the basis of the coiled-coil modeling work of Krystek et al. (1991). Starting from these models, and using the molecular modeling program ICM (Abagyan & Totrov, 1994), the wild-type sequence was mutated either to Lac28K or to Lac28E (see Figure 1 legend for sequence), respectively, in such a way that the α-helical bundles now consisted of circularly permuted and mutually interacting pairs of Lac28E and Lac28K peptides. These initial models were subjected to 400 steps of energy minimization in the side-chain torsional space employing the covalent, hydrogen-bonding, electrostatic, and solvation potential energy terms (Abagyan & Totrov, 1994). Subsequently, several independent runs of biased Monte Carlo simulation were done until the structures converged (10^6 Monte Carlo cycles at 700 °C; Abagyan & Totrov, 1994). The α-helical backbones were fixed throughout the simulation, but Monte Carlo sampling was applied to side-chain torsional degrees of freedom and the rigid body movements of the helices. No artificial constraints or forcing potentials were applied to the system. On completion of the simulation runs, 39 of the best heterotetramer conformations were retained. The original free energy function of Abagyan and Totrov (1994), including the solvation parameters and the modified-image electrostatic charges and equations, served as the Monte Carlo potential. Full details of heterotetramer modeling and Monte Carlo simulations will be described elsewhere.

Peptides, whose sequences are given in Figure 1, were synthesized on an Applied Biosystems Model 431A automated peptide synthesizer using the Boc/benzyl strategy. Peptidyl-resin was deprotected and cleaved by treatment with HF containing appropriate scavengers. Peptides were purified to homogeneity by reverse-phase HPLC (Chao et al., 1993), and their identity was established by amino acid analysis (Liu & Boykins, 1989) and electrospray or fast atom bombardment mass spectrometry analysis. Peptide concentrations were determined by one or both of the following methods: (1) quantitative amino acid analysis (Liu, & Boykins, 1989) or (2) quantitative ninhydrin analysis (Rosen, 1957).

Circular dichroism data were collected on an Aviv 62DS circular dichroism spectropolarimeter equipped with a thermoelectric device for temperature control. Spectra were

collected using a 0.5 nm step size, an averaging time of 2 s, and a bandwidth of 1.5 nm in 10 mM MOPS, pH 7.5. All other measurements were made in 10 mM MOPS, pH 7.5, at 25 °C unless stated otherwise. Data points in the thermal melts represent a time average of 3 min.

Sedimentation equilibrium experiments were performed at 25 °C in a Beckman Model XLA ultracentrifuge using an An 60 Ti rotor. Data were collected using six-channel Epon, charcoal-filled centerpieces with a 12 mm path length containing 110 μL of samples and 125 μL of buffer references. The peptide loading concentrations were 200 μM in 10 mM MOPS, pH 7.5, and 0.1 M NaCl. The density of the solvent was measured gravimetrically ($\rho = 1.0078$ g/mL). The samples were centrifuged at 20 000, 30 000, 40 000 and 50 000 rpm, and the protein absorbance was monitored at a wavelength of 242 nm. Ten successive radial scans were averaged using a 0.001 cm step size, and equilibrium was assumed if no change in distribution was observed over intervals of 2 h.

The sedimentation equilibrium data were analyzed using software from Preston Hensley (Smith Kline Beecham, King of Prussia, PA) running under Igor (Wavemetrics, Lake Oswego, OR) and incorporating the algorithm of Michael L. Johnson (Johnson et al., 1981). Partial specific volumes (Lac21E, 0.724; Lac21K, 0.776) were calculated from the weight average of the partial specific volumes of the individual amino acids (Cohn & Edsall, 1943).

RESULTS

The sequences of the peptides used here are derived from previous work (Fairman et al., 1995; Alberti et al., 1993) which showed that synthetic peptides containing the 21 C-terminal residues from Lac repressor protein could form homomeric four-chain coiled coils (or oligomeric four-helix bundles). Synthetic peptides, either 21 or 28 amino acids in length, were made in which the amino acids at all heptad positions b and c (Figure 1) are either glutamic acid (Lac21E and Lac28E) or lysine (Lac21K and Lac28K). The goals of these replacements were twofold: (1) to disfavor homotetramer formation through electrostatic repulsion and (2) to favor heterotetramer formation through specific interactions between appropriately positioned glutamic acid and lysine residues upon mixing equimolar quantities of Lac21E and Lac21K. Indeed, computer-generated models of heterotetramers confirmed the physical possibility of close interhelical interactions between charged side chains in positions b and c of the heptads (Figure 2).

Characterization of the Lac21E/K Heterotetramer. Circular dichroism (CD) spectra of either Lac21E or Lac21K alone measured at 100 μM peptide are highly characteristic of unfolded peptides showing a weak minimum at 222 nm and the indications for a second, stronger minimum below 200 nm (Figure 3). A 1:1 mixture of 50 μM each Lac21E and Lac21K reveals a spectrum of a highly helical structure containing minima of approximately the same intensity at 222 nm and at 208 nm. Similar results were obtained for the Lac28E and Lac28K peptides (data not shown), whose sequences were used for the modeling study. Experiments using Lac28E and Lac28K will be presented elsewhere.

Sedimentation equilibrium (SE) ultracentrifugation experiments were performed to determine the oligomeric states of the Lac21E and Lac21K peptides and their equimolar

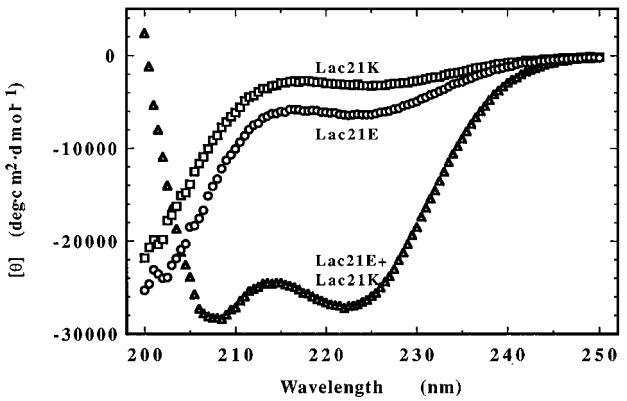

FIGURE 3: CD spectra of Lac21E and Lac21K peptides taken in 10 mM MOPS, pH 7.5, 25 °C. The concentration of total peptide is 100 μM.

mixture. Data were collected at three speeds (30 000, 40 000, and 50 000 rpm), and single species analysis of these data is presented in Table 1. Global fits to the Lac21E/K heterotetramer data, including all three speeds, using a single species model are presented in Figure 4. The molecular weights as determined either by single speed analysis or by global analysis (Table 1) are consistent with highly stable tetramer formation for the Lac21E/Lac21K peptide mixture and a largely monomeric distribution for either Lac21E or Lac21K alone.

A more quantitative estimate can be obtained of the relative stabilities of the homo- and heteromeric complexes from thermal unfolding experiments as monitored by $[\theta]_{222}$, the mean residue ellipticity at 222 nm. The T_m of a heterotetramer mixture using a total peptide concentration of 100 μM is about 75 °C (Figure 5C). This T_m depends upon peptide concentration (data not shown) as expected for an oligomerizing system; as a consequence, the ill-defined native and unfolded baselines shown in Figure 5C were more accurately determined by a global analysis of thermal unfolding curves collected at several peptide concentrations. The T_m of 75 °C reflects consideration of a native sloping baseline. The T_m's for either Lac21E or Lac21K alone, under the same conditions, are apparently well below 0 °C (Figure 5A,B). While it is not possible to calculate the T_m's for Lac21E and Lac21K, a reasonable upper limit of −20 °C results in a ΔT_m of at least 95 °C.

Having established a well-defined oligomeric state for the Lac21E/K complex, a free energy of heterotetramer formation can be measured. Thermal unfolding data for the Lac21E/K complex were fitted with the Gibbs−Helmholtz function using a method similar to that described by Thompson et al. (1993). The validity of such a two-state treatment for tetrameric coiled coils is discussed by Fairman et al. (1995). This analysis places the free energy of heterotetramer formation at −22.4 ± 1.3 kcal/mol per tetramer at 25 °C (data not shown) and corresponds to a $K_d = 4 \times 10^{-17}$ M^3 for a tetramer ↔ monomer equilibrium process. Assuming a proportionality constant of 8.0 °C/kcal for our system (O'Shea et al., 1992), the free energies of the Lac21E and Lac21K homotetramers are less than −10 kcal/mol, which corresponds to at least a 200-fold preference of heterotetramers over homotetramers.

Charge Effect on the Lac21E/K Heterotetramer Stability. The increased stability of the heterotetramer relative to the homotetramers is electrostatic in origin as judged by the

Table 1: Molecular Weight As Determined from Sedimentation Equilibrium Data

peptide	monomer	rotor speed (rpm) 30 000	40 000	50 000	global fit
Lac21E	2428	<1000	2900 ± 74%	1600 ± 41%	1700 ± 86%
Lac21K	2434	800 ± 720%	3200 ± 46%	2400 ± 32%	3100 ± 55%
Lac21E/K[a]	2431	8700 ± 12%	10200 ± 7%	9500 ± 3%	9500 ± 7%

[a] Lac21E/K represents a 1:1 mixture of Lac21E and Lac21K where the monomer molecular weight is represented as an average of the individual peptide molecular weights. The expected molecular weight for the heterotetramer would thus be 9724.

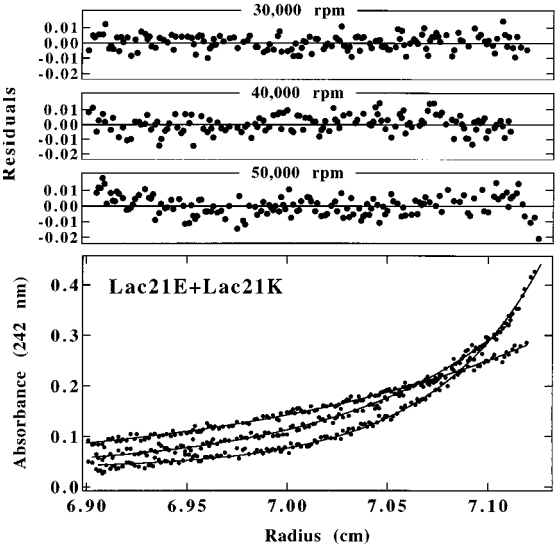

FIGURE 4: Sedimentation equilibrium analysis of the Lac21E/K complex. Total peptide concentration was 200 μM in 100 mM NaCl and 10 mM MOPS, pH 7.5, and measurements were made at 25 °C.

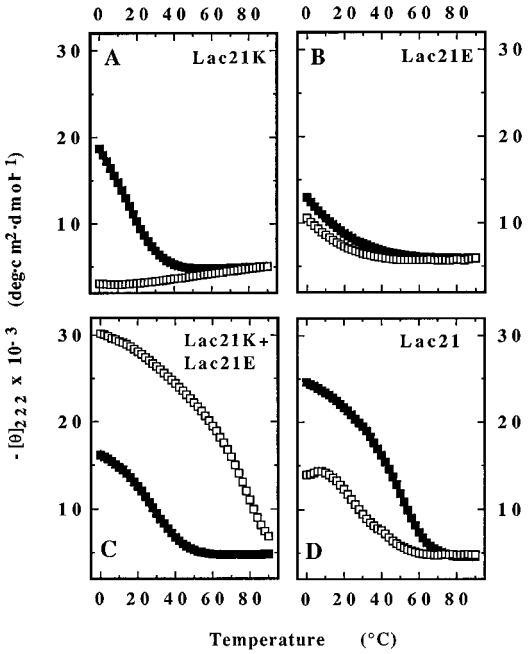

FIGURE 5: Thermal unfolding of 100 μM each (A) Lac21K, (B) Lac21E, (c) Lac21E+Lac21K, and (D) Lac21. Curves: (□) 0 M NaCl; (■) 1 M NaCl.

effects of pH on α-helix content as measured by $[\theta]_{222}$ (Figure 6). At a total peptide concentration of 50 μM, the heterotetramer state is highly populated at neutral pH, as judged by the high α-helical content. A large decrease in α-helical content is seen at acidic and basic pH's where either glutamate or lysine loses its charge, suggesting that ion pair

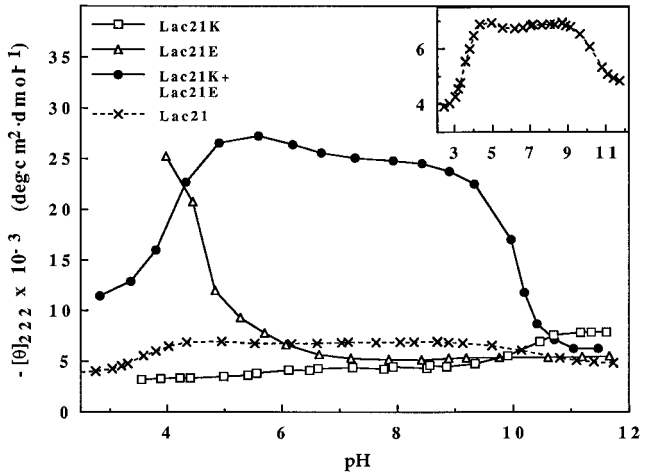

FIGURE 6: pH dependence of Lac21, Lac21E, Lac21K, and a 1:1 mixture of the two peptides. Total peptide concentration is 50 μM in 1 mM each of the sodium salts of phosphate, borate, and citrate and measurements were made at 25 °C.

interactions play an important role in heterotetramer stability. The formal charges at the ends of the peptides have been removed by acetylation of the amino terminus and amidation of the carboxyl terminus and thus would not contribute to the pH dependence of helix content (Shoemaker et al., 1987).

Both Lac21E and Lac21K appear largely unfolded at neutral pH using the same peptide concentration as that used for the Lac21E/K complex. The increased helicity of Lac21E at acid pH is caused by the removal of unfavorable charge interactions as the glutamate residues at positions b and c become protonated. Conversely, Lac21K shows added stability at alkaline pH as the lysine residues become uncharged, although the effect is much smaller. The differences in stabilities at these extreme pH's for Lac21E and Lac21K may be a combination of the intrinsic helix propensities for glutamic acid and lysine.

As a further test of the electrostatic contribution to the stability of the Lac21E/K heterotetramer, thermal unfolding in 1 M NaCl was measured by monitoring $[\theta]_{222}$. A dramatic decrease in the T_m of unfolding for the Lac21E/K complex is observed in 1 M NaCl when compared to that measured in 0 M NaCl ($\Delta T_m = 50$ °C; Figure 5C), suggesting that favorable charge−charge interactions are being screened. In contrast, 1 M NaCl greatly increases the stability of the Lac21K peptide resulting in a T_m shift from well below 0 °C to 4 °C (Figure 5A); we assume that the only effect of 1 M NaCl is to stabilize the homotetramer state. Lac21E is also stabilized by 1 M NaCl; albeit the effect is much smaller. The difference in salt effects on thermal unfolding of Lac21E and Lac21K arises from differences in their salt dependence of stability. Much higher concentrations of NaCl are required to achieve equivalent increases of tetramer stability for Lac21E as compared to Lac21K. While this phenomenon

is not well understood, three explanations can be offered to account for these differences: (1) differences in preferred rotamer distributions, (2) differences in side-chain length, and (3) differences in screening partial atomic charge densities on glutamic acid carboxyl groups and lysine amino groups.

Comparison of the Heterotetramer to the Parent Peptide, Lac21. While the Lac21E/K heterotetramer shows increased stability with respect to the individual homotetramers, the above experiments do not measure the contribution of specific interactions to the stability of the Lac21E/K heterotetramer. To learn about such specificity, the stability of the heterotetramer was compared to that of the parent peptide, Lac21 (whose sequence is given in Figure 1).

The free energy of the heterotetramer state ($\Delta G = -22.4 \pm 1.3$ kcal/mol; see above) can be compared to the free energy of the homotetramer state of Lac21 ($\Delta G = -15.7 \pm 0.2$ kcal/mol; $K_d = 3.1 \times 10^{-12}$ M^3; Fairman et al., 1995), giving an increase of 6.7 kcal/mol in stability for the heterotetramer. The difference in free energy between the heterotetramer and the homotetramer is even greater if we take into account the changes in helix propensities on going from the parent homotetramer to the Lac21E/K heterotetramer ($6.7 + 1.0 = 7.7$ kcal/mol), using the modified free energy scale of O'Neil and DeGrado (1990) (Betz et al., 1995). [Using the helix propensity scale as measured in a monomeric helix (Chakrabartty et al., 1994), a smaller adjustment of +0.48 kcal/mol to the overall free energy is required.] The average stabilizing free energy per potential glutamic acid−lysine interaction is therefore 0.6 kcal/mol.

We then asked whether the increased stability of the heterotetramer could be accounted for by favorable electrostatic interactions. A comparison of the pH dependence of α-helical content for Lac21 and Lac21E/K is shown in Figure 6. The overall stability of Lac21 is much lower than Lac21E/K at the same total peptide concentration, in good agreement with the results described above. While the magnitudes of the titration limbs for the heterotetramer are greater than that seen for Lac21, the overall shape of the pH dependences is similar (see Figure 6 inset). The small magnitudes of the titration limbs for Lac21 could be simply a consequence of the overall lack of stability at this peptide concentration. The pH dependence of the parent peptide may be due to its potential to form an *i*,*i*+4 intrahelical salt bridge between Lys 2 and Asp 6 (Marqusee & Baldwin, 1990) and an interhelical b,e′ interaction between Lys 2 and Glu 19 based on our modeling studies.

The quantitative difference in the electrostatic contributions to Lac21 and Lac21E/K stabilities was addressed by following the T_m in thermal denaturations as a function of NaCl concentration (Figure 5C,D). Recall that the ΔT_m is −50 °C for Lac21E/K on going from 0 to 1 M NaCl whereas the ΔT_m for Lac21 is about +27 °C; this is a difference in sign yielding a $\Delta\Delta T_m$ of 77 °C. The increase in stability of Lac21 in 1 M NaCl can be best explained by the well-known effect of NaCl on the hydrophobic contribution to stability. (It is unlikely that the salt effect on Lac21 is caused by screening of electrostatic repulsive interactions in light of the pH dependence of the stability of Lac21.) This result for Lac21 suggests that, while electrostatic effects may play some role in stabilizing this homotetramer (Figure 6), they are small in comparison to the hydrophobic contribution to stability.

DISCUSSION

Our goal was to design heterospecific four-chain coiled coils using the Lac peptide model system that we have previously described (Fairman et al., 1995). We had two aims in mind: (1) to assess determinants of specificity for helix-pairing interactions and (2) to develop a model heterooligomeric scaffolding system for the design of multifunctional/multivalent fusion proteins. The work presented here addresses the first aim.

Toward the first aim, a modeling study was initiated to identify amino acids which, when placed into specific heptad positions, would result in stabilizing heterospecific interactions. Both antiparallel and parallel symmetries of the chains in the heterotetramer were built. As we have observed in our previous modeling studies (Fairman et al., 1995), parallel and antiparallel symmetries are equally plausible. Experimental evidence was provided for the antiparallel arrangement of homotetrameric coiled coils based on the Lac sequence by Fairman et al. (1995) and later confirmed by the X-ray structure of the tetramerization domain from Lac repressor (Friedman et al., 1995). Therefore, by inference we believe that the heterotetramers are antiparallel as well. We are actively pursuing experiments to measure directly the symmetry of the heterotetramers.

Inspection of both parallel and antiparallel models suggested that putting glutamic acid at all b and c heptad positions of one chain and lysine at equivalent positions in a second chain would provide the correct stereochemistry to form specific interhelical hydrogen-bonding interactions regardless of the symmetry of the coiled coils. Figure 2 shows the interactions between the glutamates and lysines only for the antiparallel arrangement of coils. Although these positions are close enough to allow for side-chain interactions, they do not require any burial of the charges and therefore should not affect the oligomeric state. It is also possible to form both heterotrimers (Nautiyal et al., 1995) and heterotetramers (Lumb & Kim, 1995a) whose specificity is defined by charged residues at e and g positions in reengineered GCN4 derivatives. However, we wanted to avoid these positions in our studies since charged residues at these positions can also mediate the oligomerization state in GCN4 (Alberti et al., 1993) and in another coiled-coil system (Krylov et al., 1994).

Synthetic peptides (Lac21E and Lac21K), incorporating charged residues at b and c positions, strongly favor heterotetramer formation over homotetramer formation. More importantly though, from the viewpoint of protein design, the Lac21E/K heterotetramer is stabilized directly by glutamate−lysine ion pair interactions. This is demonstrated by measuring the difference in the stability between the Lac21E/K heterotetramer and its parent peptide, Lac21 ($\Delta\Delta G = -7.7$ kcal/mol). This difference in free energy takes account of the difference in intrinsic helix propensities upon substitution of the native residues for glutamate and lysine using the modified scale of O'Neil and DeGrado (1990) (Betz et al., 1995). The strengths of these specific interactions are dominated by electrostatics as shown by comparative pH and NaCl experiments using Lac21E/K and Lac21 (Figures 5 and 6). Such experiments have been used before to demonstrate the formation of ion pair interactions between glutamate and lysine in designed monomeric α-helical systems (Marqusee & Baldwin, 1987; Merutka & Stellwagen, 1991; Scholtz et al., 1993).

There are two other examples of specific electrostatic interactions that stabilize coiled coils, both in heterodimers. Graddis et al. (1993) showed that synthetic peptides containing the heptad repeats $(VSSLESE)_3$ and $(VSSLKSK)_3$ have a pH dependence of heterodimer stability similar to what we have observed (Figure 6). In a more quantitative study, Vinson and co-workers (Krylov et al., 1994) showed a coupling energy of −1.14 kcal/mol for glutamate−arginine pairs in e and g′ positions of a heterodimeric coiled coil. This coupling energy is a consequence of a specific interaction between these two amino acids although the role of electrostatics is as yet unclear (Vinson, personal communication).

Work from several laboratories suggests that another mechanism for conferring stability in heteromeric coiled coils is to disfavor homomeric interactions through charge repulsion, rather than to form specific ion pair interactions (O'Shea et al., 1993; Zhou et al., 1994; Lumb & Kim, 1995b). The lack of pH dependence in the stability of the "Velcro" heterodimers studied by Kim and his colleagues (O'Shea et al., 1993) led them to conclude that charge repulsion played a dominant role in heterospecificity, a concept referred to as "negative design". It is perhaps not surprising in light of the thermodynamic analysis from Vinson and co-workers (Krylov et al., 1994) which showed that glutamate−lysine pairs contribute only −0.14 kcal/mol of coupling energy at these same positions in a related model system.

For the purposes of protein design, it will be advantageous to find side-chain interactions defining heterospecificity in a direct and "positive" manner. For example, while the fos and jun system may utilize "negative design" principles to preferentially form heterodimers rather than homodimers (O'Shea et al., 1989), other heteromeric interactions are observed (Glover & Harrison, 1995) that may help to further define specificity and stability and, thus, further minimize the potential to form nonspecific interactions with other coiled coils present *in vivo*.

Toward our second aim, it is clear that an understanding of the contributions electrostatics and hydrophobics make to stability and specificity in coiled coils will play an important role in the design of *in vivo* model systems utilizing these motifs as oligomerization scaffolding domains. Studies of the Lac28E and Lac28K sequences, either in isolation or as fusions to other proteins, under diverse physiological conditions are currently underway to find out how stable our designed heterotetramers are in a complicated *in vivo* milieu. We envision using such oligomerization scaffolds to bring together heterologous functions such as combining different antibody specificities in a single complex (Renner et al., 1994; Mallender et al., 1994).

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