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Surface salt bridges stabilize the GCN4 leucine zipper

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Abstract

We present a study of the role of salt bridges in stabilizing a simplified tertiary structural motif, the coiled-coil. Changes in GCN4 sequence have been engineered that introduce trial patterns of single and multiple salt bridges at solvent exposed sites. At the same sites, a set of alanine mutants was generated to provide a reference for thermodynamic analysis of the salt bridges. Introduction of three alanines stabilizes the dimer by 1.1 kcal/mol relative to the wild-type. An arrangement corresponding to a complex type of salt bridge involving three groups stabilizes the dimer by 1.7 kcal/mol, an apparent elevation of the melting temperature relative to wild type of about 22 °C. While identifying local from nonlocal contributions to protein stability is difficult, stabilizing interactions can be identified by use of cycles. Introduction of alanines for side chains of lower helix propensity and complex salt bridges both stabilize the coiled-coil, so that combining the two should yield melting temperatures substantially higher than the starting species, approaching those of thermophilic sequences.

Keywords: GCN4; leucine zipper; salt bridge; thermal stability

Interactions that stabilize the native state of proteins include the hydrophobic effect, van der Waals interactions, hydrogen bonds and ionic effects, including dipole interactions and salt bridges (Creighton, 1993). The question of which of these are most important in protein stabilization has been debated since the review by Kauzmann (1959). One aspect of the problem concerns how to account for the additional stabilization of proteins from thermophiles, which can have very high thermal stabilities (see Hiller et al., 1997). Since the pioneering work of Matthews et al. (1974) on thermolysin, structures of thermophilic and mesophilic proteins have been compared in a search for clues to what accounts for the higher stability of the former (Korndörfer et al., 1995; Yip et al., 1995; Hatanaka et al., 1997; Robb & Maeder, 1998). In 1978, Perutz (1978) observed that the main discernible difference between a thermophilic and mesophilic version of ferrodoxin lay in the greater number of salt bridges on the surface of the thermophile. As more crystal structures of thermophilic proteins have become available, other mechanisms have been proposed to explain their stability (Vogt & Argos, 1997): improved internal packing, burial of a greater hydrophobic area (Chan et al., 1995; Delboni et al., 1995), and networks of complex salt bridges (Yip et al., 1995; Pappenberger et al., 1997). While individual surface salt bridges contribute moderately to protein stability, with values about 0.5 kcal/mol or less (Dao-Pin et al., 1991), interactive combinations of salt bridges referred to as complex salt bridges may be more potent (Horovitz et al., 1990; Yip et al., 1995; Robb & Maeder, 1998).

Here, we consider the role of complex salt bridges in stabilizing a simplified model protein structure. The strength of a salt bridge can be estimated by different experimental methodologies: changes in the helicity of model peptides (Merutka & Stellwagen, 1990; Lyu et al., 1992; Scholtz et al., 1993), shifts in pK_a of interacting side chains (Anderson et al., 1990; Lumb & Kim, 1996), or T_m differences in model proteins (Horovitz et al., 1990; Dao-Pin et al., 1991). Using the first method, Smith and Scholtz (1998) report that simple salt bridges stabilize helical peptides by free energies ranging from 120 cal/mol for DK in an $i, i + 3$ spacing to 650 kcal/mol for HE with an $i, i + 4$ spacing. In α helices, the $i, i + 4$ spacing is a stronger stabilizing interaction than the $i, i + 3$ spacing (Huyghues-Despointes et al., 1993a). In salt bridges between E and K side chains, the directionality EK vs. KE (N to C terminus) does not seem to exert a major influence on the stability. Interactions between charged side chains with the helix dipole, however, may influence the stabilizing effect of salt bridges (Huyghues-Despointes et al., 1993b).

Anderson et al. (1990) studied a single buried salt bridge that stabilized T4 lysozyme by more than 4 kcal/mol. This salt bridge is located within the hydrophobic core of the molecule. By contrast, engineered surface salt bridges in the same protein are much less stabilizing (Dao-Pin et al., 1991). The minor extent of stabilization of surface bridges they observe has been ascribed to helix-dipole or side-chain to side-chain interactions other than salt bridges. Dahiyat et al. (1997) employed an algorithm that quantitatively assesses side-chain to side-chain interactions to modify helix sur-

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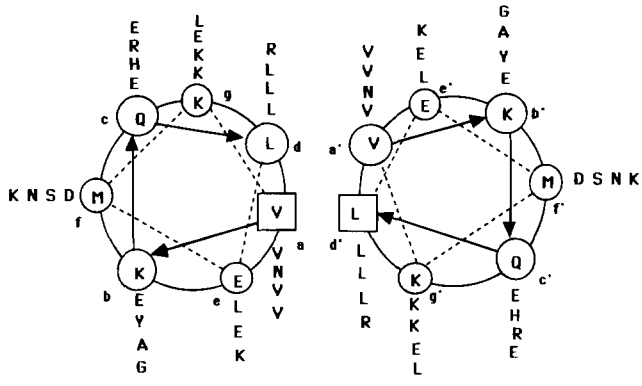
faces. They concluded that hydrogen bonds involving surface residues do not contribute significantly to the stability of their protein model. Horovitz et al. (1990) used mutant thermodynamic cycles to measure the strength of a naturally occurring complex salt bridge linking Asp8, Asp12, and Arg110 in barnase. They report a stabilization energy of 1.25 and 0.98 kcal/mol for the two Asp-Arg salt bridges. If the salt bridges are uncoupled, the energy of the bridges drops by 0.77 kcal/mol, indicative that strong interactions connect the three charged groups. Free energy calculations by Hendsch and Tidor (1994) indicate that buried salt bridges may be destabilizing relative to hydrophobic groups, because of the requirement for solvation changes between ionic side chains within the hydrophobic core. Salt bridges that form networks could still be stabilizing according to this level of theory. Waldburger et al. (1995) demonstrated directly that substituting a buried salt bridge network by hydrophobic residues stabilizes the protein, in line with these calculations.

To investigate the effect of solvent exposed salt bridges on protein stability, we use the GCN4 leucine zipper, a coiled coil structure consisting of two 34 amino acid α helices that form a dimer. The crystal structure has been determined (O'Shea et al., 1991), the model has been analyzed thermodynamically (Thompson et al., 1993; D'Aquino et al., 1996) and used as a test bed for protein design (Dahiyat et al., 1997) and combinatorial mutagenesis experiments (Hu & Sauer, 1992; Hu et al., 1993). GCN4 consists of four heptad repeats of the form $(\mathbf{abcdefg})_n$—in which most **a** and **d** residues are aliphatic amino acids that comprise the hydrophobic core of the duplex (Fig. 1)—with an additional partial copy. While GCN4 is a dimer, its stoichiometry depends on the presence of different side chains at the **a** and **d** positions (Harbury et al., 1993). The **e** and **g** residues interact with the opposite strand and influence both stoichiometry and stability of coiled coils (Hu & Sauer, 1992; Lavigne et al., 1996; Lumb & Kim, 1996). Frequently the side chains at these positions can form salt bridges (O'Shea et al., 1991). The parallel or antiparallel orientation of the two strands in the dimer is determined by a single Asn residue at an **a** position that hydrogen bonds to the corresponding Asn of the second chain (Lumb & Kim, 1995).

Residues at the solvent exposed **b**, **c**, and **f** positions do not appear to be essential for maintaining the coiled coil structure, and have been selected to engineer variants for analysis of stability differences (O'Neil & DeGrado, 1990; Dahiyat et al., 1997). The conformation of the hydrophobic core appears to be relatively insensitive to mutation of the **b**, **c**, or **f** residues, so that changes in stability at these sites can arguably be attributed to contributions from local effects and not rearrangements within the hydrophobic core. The sequence of GCN4 is shown in Figure 1. The **a** and **d** residues are indicated in bold face. For purposes of this study they are fixed, as are the **e** and **g** residues. The **b**, **c**, and **f** sites are substituted to introduce alanines, simple or complex salt bridges. The simplest salt bridge that can be introduced would be a pair of side chains such as ER spaced at an interval $(i, i + 4)$. E is preferred to D because of its more favorable helix propensity, while R is preferred to K because it has a larger positive surface that favors complex salt bridging (Musafia et al., 1995). A K → R substitution pattern has been identified in thermophilic proteins (O'Fagain, 1995). Starting from this canonical salt bridge, we form a network involving exclusively the **b**, **c**, and **f** sites. The mutations of this study are shown in Figure 1.

As a benchmark to evaluate effects of helix propensity, a series of alanines is used. Alanine has the highest helix propensity in



heptad repeat:	a	b	c	d	e	f	g	a	b	c	d	e	f	g	a
GCN4	V_{10}	E	E	L	L	$\underline{S}_{15}$	K	N	Y	$\underline{H}$	L_{20}	E	$\underline{N}$	E	V

AAA	A	A	A
AAR	A	A	R
RAA	R	A	A
RAR	R	A	R
AEA	A	E	A
REA	R	E	A
AER	A	E	R
RER	R	E	R
SRE	S	R	E

Fig. 1. Helical wheel representation of GCN4 and the mutations of this study. The model system used has an additional Lys and Val and no Arg at the N-terminus, and is otherwise identical to GCN4-p1 (Lumb et al., 1994).

several peptide models (Rohl et al., 1996; Yang et al., 1997), at the **f** position in coiled coils (O'Neil & DeGrado, 1990) and at exposed helical sites in proteins (Blaber et al., 1993; Myers et al., 1997). The latter studies make it clear that helix stability is directly coupled to that of the native state, making it logical to optimize secondary structure stabilization in attempts to enhance protein stability (Menendez-Arias & Argos, 1989). Substituting side chains with higher helix propensity offers one approach to enhancing protein thermostability (Warren & Petsko, 1995; Vogt & Argos, 1997). In this study, we correct for propensity effects, using the free energy scale of O'Neil and DeGrado (1990), which is based directly on substitutions at external sites, to assess electrostatic and other interactions in a coiled coil model.

Results and discussion

The CD spectra of the mutations of GCN4 employed in this study show minima at 222 and 208 nM, characteristic of an α helix (not shown). All mutants employed in this study show similar CD spectra indicative of 90% plus helix content. The stability of each protein was monitored by recording profiles of $[\theta]_{222}$ as a function

of temperature for samples with a fixed monomer concentration, 10 mM in 150 mM NaCl and 50 mM phosphate at pH = 7. The melting behavior of WT, AAA, and RER is shown in Figure 2. Each shows the cooperative unfolding anticipated for a coiled coil-random coil transition (O'Neil & DeGrado, 1990). The transitions are analyzed thermodynamically in terms of a two-state model (Thompson et al., 1993). All melting experiments are reversible and the proteins retain 80–90% of their helicity after the melt. Analytical ultracentrifugation of the RER mutant reveals essentially a pure dimer species (Fig. 3). Since no residues directly responsible for oligomerization have been altered, it can be assumed that all mutants are dimeric (Harbury et al., 1993).

The melting temperatures for GCN4 solutions of the same concentration are related to ΔG using the modified Gibbs–Helmholtz equation (Thompson et al., 1993):

$$\Delta G_i^0 = \Delta H_i^0(T_i^0) + \Delta C_{p,i}(T - T_i^0) - T[\Delta S_i^0(T_i^0) + \Delta C_{p,i} \ln(T/T_i^0)]. \quad (1)$$

Thompson et al. (1993) determined the following parameters for the wild-type GCN4 zipper in low salt at pH = 7, using DSC (all expressed per mole monomer):

$$\Delta H_i^0(T_i^0) = 34.5 \text{ kcal/mol} \quad (C_T = 10\ \mu\text{M}) \quad (2)$$

$$\Delta S_i^0(T_i^0) = 93.9 \text{ cal/(K mol)} \quad (C_T = 10\ \mu\text{M}) \quad (3)$$

$$\Delta C_{p,i} = 135 \text{ cal/(K mol)} \quad (C_T = 504\ \mu\text{M}). \quad (4)$$

As a reference for stability, the version substituted with alanines at the sites of interest AAA is used. Alanine has the highest helix propensity in coiled coils (O'Neil & DeGrado, 1990) and helical sites on the surface of proteins (Blaber et al., 1993) and has a limited ability to interact with neighboring side chains (Creamer & Rose, 1995; Rohl et al., 1996). The AAA mutation raises the melting temperature of the dimer over that of the WT by 14 °C equivalent to 1.07 kcal/mol monomer (Thompson et al., 1993). All values are given in kcal per mole monomer rather than dimer following Thompson et al. (1993). The stabilization of 14 °C then reflects the favorable helix propensity of alanine relative to H, S, and K in the wild-type, including any other interactions (e.g., H-bonds, packing, structural relaxation) that are altered. Table 1 summarizes the main results of this study, showing the T_m of each protein, the free energy differential with respect to wild-type, the estimated free energy due to propensity alone and the residual free energy, including the salt bridge or any other effect.

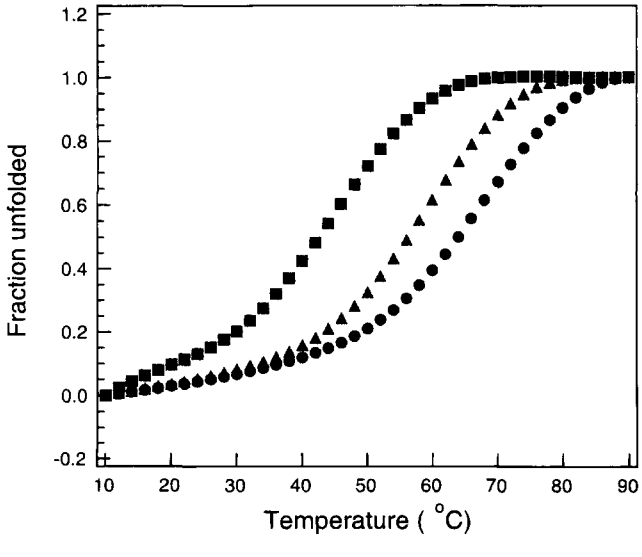


Fig. 2. Thermal unfolding profiles for the WT (■), AAA (▲), and RER (●) peptides at 10 μM concentration in PBS buffer. The data are measured as $[\theta]_{222}$ values, normalized to the low and high temperature values.

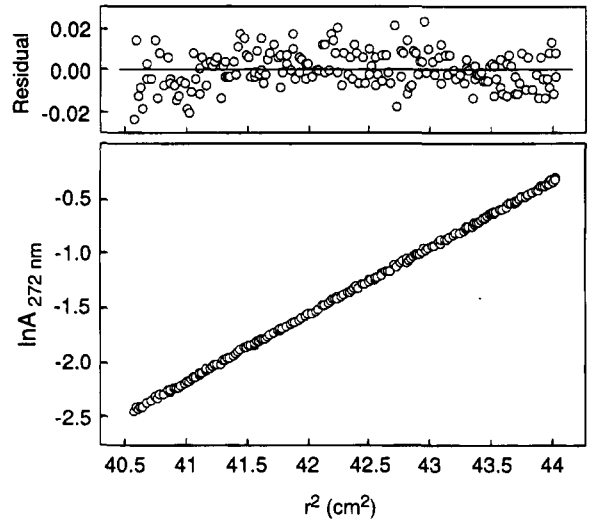


Fig. 3. Representative sedimentation equilibrium data for RER (300 μM) in PBS buffer. The random distribution of the residuals indicates that the data fit well to an ideal single-species model.

Table 1. *Thermodynamic analysis of the GCN4 mutants*[a]

Mutant	T_m[b] (°C)	$\Delta\Delta G_{WT}$[c] (kcal/mol)	$\Delta\Delta G_{Ala}$[d] (kcal/mol)	Propensity penalty[e] (kcal/mol)	Residual interaction[f] (kcal/mol)
RER	67	−1.72	−0.65	0.75	−1.4
REA	66	−1.64	−0.57	0.65	−1.22
AAR	61	−1.23	−0.16	0.1	−0.26
RAR	60	−1.15	−0.08	0.2	−0.28
AER	60	−1.15	−0.08	0.65	−0.73
RAA	59	−1.07	0	0.1	−0.1
AAA	59	−1.07	0	0	0
AEA	55	−0.76	0.31	0.55	−0.24
SRE	48	−0.22	0.85	1.1	−0.25
SHN (WT)	45	0	1.07	1.8	−0.73

[a] All data are per monomer.

[b] The melting temperatures were obtained by taking the derivative of $[\theta]_{222}$ vs. temperature.

[c] $\Delta\Delta G_{WT} = \Delta G_{MUT} - \Delta G_{WT}$; $\Delta\Delta G_{Ala} = \Delta G_{MUT} - \Delta G_{AAA}$; ΔG_{MUT} and ΔG_{WT} were obtained according to the formula $\Delta G_i^0 = \Delta H_i^0(T_i^0) + \Delta C_{p,i}(T - T_i^0) - T[\Delta S_i^0(T_i^0) + \Delta C_{p,i} \ln(T/T_i^0)]$ (Thompson et al., 1993).

[d] Propensity penalty = $S_{MUT} - S_{Ala}$ (O'Neil & DeGrado, 1990). Propensities have been temperature adjusted.

[e] Residual interaction = $\Delta\Delta G_{Ala}$ − propensity penalty.

Introduction of a simple salt bridge ER (AER) increases the T_m by only 1 °C relative to AAA, a stabilization of only 0.08 kcal/mol. To understand the low apparent stabilizing energy of this salt bridge, the helix propensities of the substituted glutamic acid and arginine side chains relative to Ala must be taken into account. While the propensity scale derived from substitutions at **f** sites in a coiled coil (O'Neil & DeGrado, 1990) does not agree exactly with other scales, the deviations in ΔG are small (Yang et al., 1997). Substitution of E → A and R → A should reduce the stability of the monomer by 0.65 kcal/mol in terms of propensity (O'Neil & DeGrado, 1990). Other interactions complicate parsing the free energy. Structural relaxation can occur in a coiled coil, despite the simplicity of its fold. Local changes in structure on substituting side chains of different size or polarity are hard to avoid or predict in the absence of high resolution structural data (Blaber et al., 1994). Interactions between charged or bulky hydrophobic residues, for example, are likely to play a role (Creamer & Rose, 1995). One approach is to analyze the salt bridge in terms of mutant cycles (Serrano et al., 1990). Figure 4A illustrates the mutant cycles applicable to the AER substitution. The measured free energy changes are indicated along the sides of the square; in each case, it is assumed the free energy differences apply to the native state itself rather than to the unfolded states. We have corrected the free energies for the effect of propensity of the introduced residues. For example, the change AAA → AEA results in a destabilization of 0.31 kcal/mol. Replacing an alanine by a glutamic acid entails a propensity penalty of 0.55 kcal/mol. This implies a residual stabilizing interaction of 0.24 kcal/mol. The stabilization of the AER salt bridge is only

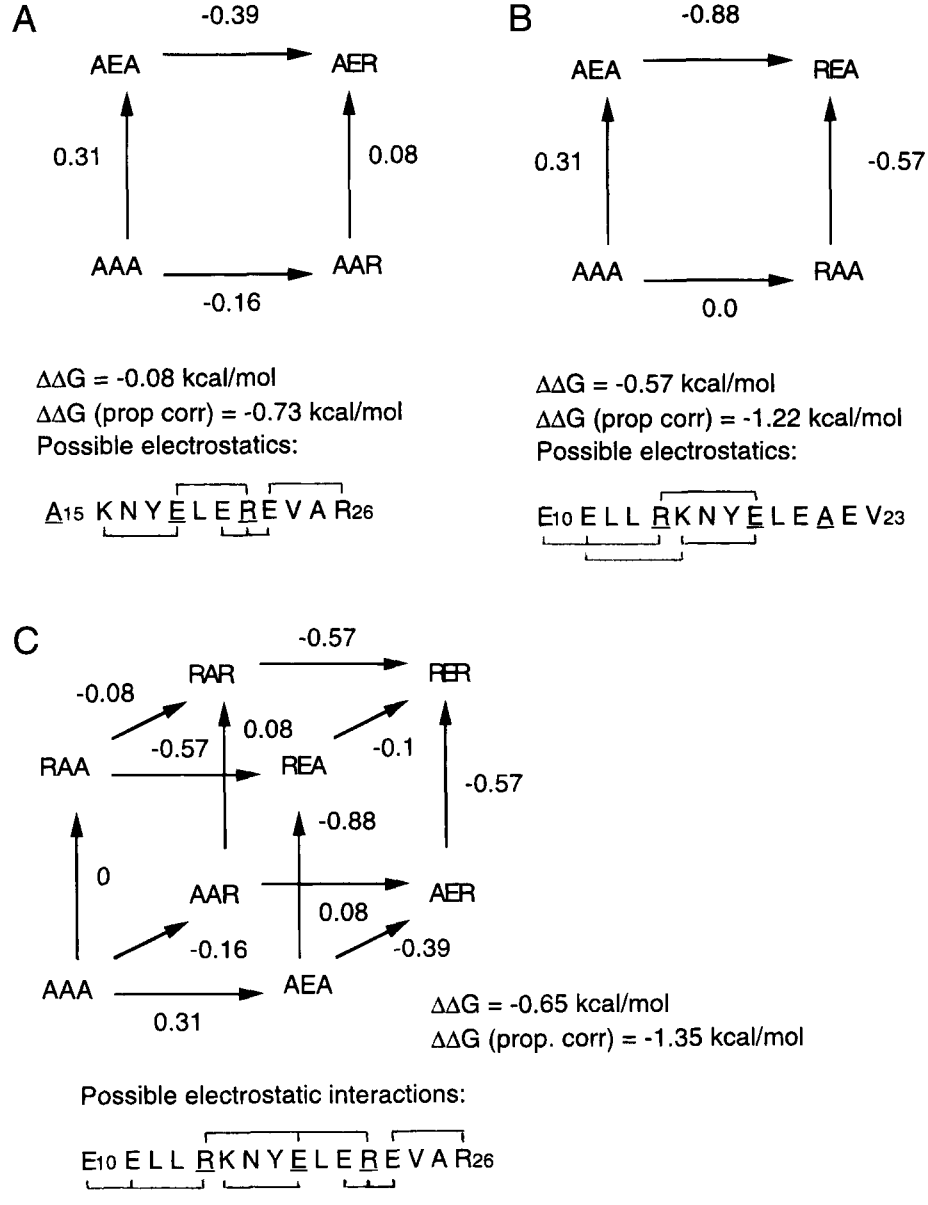


Fig. 4. Alanine mutant cycles for (**A**) AER, (**B**) REA, and (**C**) RER mutants of GCN4. $\Delta\Delta G$ (prop corr) = $\Delta\Delta G - \Sigma(S_{mut} - S_{Ala})$, the free energy values corrected for helix propensity differences according to O'Neil and DeGrado (1990).

0.08 kcal/mol as compared to AAA. If propensities are taken into account, the salt bridge is stabilizing by 0.73 kcal/mol and slightly greater than the stabilization observed by introducing simple salt bridges in helical peptides (Lyu et al., 1992; Smith & Scholtz, 1998). This implies residual interactions, possibly electrostatic in origin, as shown at the bottom of the panel.

The potential complex salt bridge introduced via the RER mutant is analyzed in terms of cycles in Figure 4C. Each of the two arginines in RER, in principle, can form a salt bridge with the glutamic acid in the middle position—one of the patterns detected by Yip et al. (1995). The RER mutant has a melting temperature 22 °C higher and is 1.72 kcal/mol more stable than the WT and 0.65 kcal/mol more stable than AAA. If the helix propensities of the different residues are taken into account the residual stabilization is 1.4 kcal/mol. Can this stabilization be ascribed to the complex salt bridge exclusively? To determine this we follow the mutant cycles in Figure 4. The single AEA and RAA substitutions show slightly different free energy effects. The RAA mutant has the same stability as AAA and a small favorable residual interaction of −0.1 kcal/mol. This may be due to an electrostatic interaction between the Arg and two glutamic acid residues upstream. The single salt bridge in REA is stabilized by 0.57 kcal/mol, more than in AER. The residual stabilization is 1.22 kcal/mol, which is exceptionally large. Again, it is likely that interactions other than formation of the simple salt bridge contribute to this value, with significant electrostatic effects. The mutant RAA shows a residual stabilization of 0.1 kcal/mol, while AEA is stabilized by 0.24 kcal/mol, a total stabilization of 0.34 kcal/mol. The difference—REA (1.22 kcal/mol) − (RAA + AEA) (0.34 kcal/mol) = 0.88 kcal/mol—can therefore be attributed to the new salt bridge, the effect of multiple interactions introduced by RE. The overall residual stabilization of RER amounts to 1.4 kcal/mol, slightly above that in REA and the sum of interactions in REA and AAR combined. The overall stability can be clearly enhanced by expansion of the electrostatic network, as predicted by several calculations (Nakamura, 1996).

Introducing a simple salt bridge stabilizes to different extents as shown in the case of SRE. The serine has a lower helix propensity than alanine. There is then a residual destabilization of 0.25 kcal/mol, which is much lower than the stabilization by REA (1.22 kcal/mol). The destabilization caused by the introduction of this salt bridge may be ascribed to the presence of three sequential glutamic acids. Interactions other than a simple salt bridge, either hydrophobic or electrostatic, with neighboring residues cannot be excluded of course.

To assign self-consistent values to the stabilization free energies of individual salt bridges is not straightforward, even in the simplified tertiary structural background of a coiled coil. Correcting the newly introduced residues for their helix propensities and using cycles allows estimation of their side-chain interactions and/or other residual effects. The salt bridge patterns we have studied stabilize the coiled coil significantly relative to the alanine reference. One simple salt bridge (REA) stabilizes by 1.64 kcal/mol relative to the WT. The stabilization compared to the alanine mutant is 0.57 kcal/mol. The putative complex RER bridge is stabilizing by 1.72 kcal/mol; the coupling free energy (Horovitz et al., 1990) is large for the central E in RER: 0.88 kcal/mol, calculated from the difference in free energies between RER and RAR relative to AEA and AAA (Fig. 4C). This is greater than the values determined by Krylov et al. (1998) for salt bridges at the **e** and **g** sites in a coiled coil, although the number must be interpreted with care. First, the pH and salt dependence have not been measured to isolate the electrostatic contribution more definitively (Krylov et al., 1998). Second, in GCN4, a chain of electrostatic interactions including charged side chains at **e** and **g** sites can modulate the interactions among the charges in RER, as suggested in Figure 4. Third, structural relaxation can play a role and is not easy to assess. Cases in which methylenes of a side chain change orientation or position only slightly, for example, might escape detection by NMR shifts that are relatively insensitive to changes in this region of the spectrum (Horovitz et al., 1990). X-ray structure analysis remains the method of choice (Blaber et al., 1993, 1994).

While local vs. nonlocal interactions are hard to discriminate, electrostatics arguably play a significant role in the stabilizing effect of RER. To put the conclusion in another way, salt bridges at exposed helix sites can stabilize coiled-coil structure significantly: Substitution of only 3 of the 34 amino acids in each monomer in RER yields an overall stabilization of 1.72 kcal/mol per monomer relative to WT. Replicating the T_m increase of 22 °C due to a single bridge of complex type suggests that networks of such bridges can contribute significantly to the stability of thermophiles (Yip et al., 1995). However, in any such proposal one needs to verify that the stabilizing interactions remain effective beyond 100 °C (Hiller et al., 1997) and that the additional free energy contributions remain additive through this extreme temperature range.

Materials and methods

Cloning and mutagenesis

Plasmid pRER, encoding the mutant GCN4 RER protein, was constructed by oligonucleotide-directed mutagenesis (Kunkel et al., 1987) of plasmid p1 (Lumb et al., 1994). Plasmids for the expression of the alanine mutant peptides were derived sequentially from pRER. Standard recombinant DNA techniques were used (Sambrook et al., 1989).

Peptide synthesis and purification

All recombinant peptides were expressed in *Escherichia coli* BL21(DE3) pLysS using the T7 expression system (Studier et al., 1990). Cells, freshly transformed with an appropriate plasmid, were grown to late log phase. Protein expression was induced by addition of 0.5 mM isopropylthio-β-D-galactoside (IPTG). After another 3 h of growth at 37 °C, the bacteria were harvested by centrifugation, and the cells were lysed by glacial acetic acid. All peptides were purified from the soluble fraction to homogeneity by reverse-phase high-performance liquid chromatography (HPLC), using a Vydac C-18 preparative column and a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid (TFA). The identity of each HPLC-purified peptide was confirmed by laser desorption mass spectrometry. In all cases, the observed and expected molecular masses agreed to within 0.1% of the calculated peptide mass. Peptide concentrations were determined by absorbance at 280 nM in 6 M GuHCl (Edelhoch, 1967).

CD spectroscopy

Circular dichroism (CD) spectra were recorded on an AVIV Model 62DS CD spectrometer equipped with a thermoelectric sample temperature controller. Samples for wavelength spectra were

10 μM peptide in 150 mM NaCl and 50 mM sodium phosphate, pH 7.0 (PBS buffer). The cuvette was 0.1 cm in pathlength. The wavelength dependence of molar ellipticity, $[\theta]$, was monitored at 4 °C as the average of three scans, using a 5 s integration time at 1.0 nM wavelength increments. Spectra were baseline-corrected against the cuvette with buffer alone. Helix content was estimated from the CD signal by dividing the mean residue ellipticity at 222 nM by the value expected for 100% helix formation by helices of comparable size, $-33{,}000$ deg cm^2 dmol^{-1} (Yang et al., 1986).

Samples for thermal unfolding studies contained 10 μM peptide in PBS buffer. A 1.0 cm pathlength cell was used with continuous stirring. Thermal stability was determined by monitoring the change in CD signal at 222 nM as a function of temperature, and thermal melts were performed in 2° intervals with a 2 min equilibration at the desired temperature, and an integration time of 30 s. All melts were reversible. The midpoint of the thermal unfolding transition (T_m) was determined from the maximum of the first derivative, with respect to the reciprocal of the temperature, of the $[\theta]_{222}$ values (Cantor & Schimmel, 1980). The error in estimation of T_m is ± 1 °C.

Sedimentation equilibrium

Apparent molecular weights were determined by sedimentation equilibrium with a Beckman XL-A Optima analytical ultracentrifuge at 20 °C. Samples were dialyzed against the reference buffer (PBS) for at least 12 h. Samples with initial peptide concentrations of 150, 300, and 600 μM were analyzed at rotor speeds of 35,000 or 38,000 rpm. Data sets (six per peptide) were fitted simultaneously to a single-species model with the program NONLIN (Johnson et al., 1981) to yield an apparent sedimentation constant. Specific volumes and solvent densities were calculated as described by Laue et al. (1992).

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