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Mechanism of Specificity in the Fos-Jun Oncoprotein Heterodimer

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Summary

Fos and Jun, the protein products of the nuclear proto-oncogenes c-*fos* and c-*jun*, associate preferentially to form a heterodimer that binds to DNA and modulates transcription of a wide variety of genes in response to mitogenic stimuli. Both Fos and Jun contain a single leucine zipper region. Previous studies have shown that the leucine zippers of Fos and Jun are necessary and sufficient to mediate preferential heterodimer formation. The leucine zipper regions from Fos and Jun are also known to fold autonomously, most likely as two-stranded, parallel coiled coils. We show here that 8 amino acids from Fos and from Jun are sufficient to mediate preferential heterodimer formation in a background of the GCN4 leucine zipper sequence. Using pH titration and amino acid replacements, we also show that destabilization of the Fos homodimer by acidic residues provides a major thermodynamic driving force for preferential heterodimer formation.

Introduction

Specificity in protein–protein interactions is essential for almost all biological processes. Interactions between proteins are difficult to study and understand because the molecules involved are often large and complex. The interacting surfaces of proteins may be composed of amino acids that are distant in primary sequence, making it difficult to map residues important for the interaction. Additionally, because specificity is determined by the relative thermodynamic stabilities of all possible protein–protein interactions, understanding this process requires identifying forces that stabilize preferentially the favored complex as well as forces that destabilize the incorrect one.

One well-characterized protein–protein interaction is the Fos–Jun heterodimer. Fos and Jun, the protein products of the nuclear proto-oncogenes c-*fos* and c-*jun*, bind to DNA as dimers and modulate transcription of a wide variety of genes in response to mitogenic stimuli (for reviews of Fos and Jun, see Curran and Franza, 1988; Ransone and Verma, 1990). While the Jun protein forms a stable homodimer that can bind to DNA, the Fos protein does not bind DNA detectably because it does not dimerize well. The Fos and Jun proteins preferentially form a heterodimer that is thought to be the predominant species in many cell types (Franza et al., 1987; Curran et al., 1985). Deletion analyses and mutagenesis studies have demonstrated that a ~35 amino acid leucine zipper region (Landschulz et al., 1988) from Fos and from Jun is necessary for preferential heterodimer formation (Ransone and Verma, 1990).

A peptide corresponding to the leucine zipper region from GCN4, a yeast transcriptional activator, has been shown to form a two-stranded, parallel coiled coil (O'Shea et al., 1989a, 1991; Oas et al., 1990; Rasmussen et al., 1991). The two-stranded, coiled-coil motif was proposed by Crick (1953) and consists of two right-handed α helices wrapped around one another with a slight left-handed superhelical twist. Peptides corresponding to the leucine zipper regions from Fos and from Jun fold as parallel, helical dimers, suggesting that they also belong to the two-stranded coiled-coil family (O'Shea et al., 1989b). Additionally, the Fos and Jun leucine zipper peptides preferentially form a heterodimer, demonstrating that these sequences are sufficient to mediate preferential heterodimer formation (O'Shea et al., 1989b).

The Fos and Jun leucine zipper peptides provide a simple system for studying the specificity underlying a protein–protein interaction: two helices that prefer to interact with each other rather than with themselves. The structure of both components of this system is simple, and a relatively small region of primary sequence contains the determinants for specific heterodimer formation (O'Shea et al., 1989b). Furthermore, the preferential interaction between the Fos and Jun leucine zipper peptides mimics the specificity seen with the intact proteins (Smeal et al., 1989).

The goal of this study is to understand the mechanism of specificity in the Fos–Jun peptide system and to outline the basis for preferential heterodimer formation in both structural and energetic terms.

We demonstrate that 8 amino acids from Fos and from Jun are sufficient to mediate preferential heterodimer formation. Furthermore, the thermodynamic driving force for preferential heterodimer formation appears to be destabilization of the Fos homodimer by acidic residues. The charged residues in the Fos leucine zipper that are important for specificity are those known to be adjacent to the hydrophobic interface of coiled-coil proteins.

Results

pH Dependence of Stability in the Fos and Jun Leucine Zipper Peptides

The relative stabilities of the Fos and Jun peptide heterodimer and homodimers (Table 1) indicate that the Fos peptide homodimer is substantially less stable than the Fos–Jun heterodimer and the Jun homodimer (O'Shea et al., 1989b). The Fos and Jun peptides used in the studies described here differ slightly from those used previously (O'Shea et al., 1989b); the new peptides are shorter, lacking both an N-terminal residue and 7 amino acids following

Table 1. T_m for Various Disulfide-Bonded Peptide Pairs

Peptide Pair (Disulfide Bonded)	T_m(°C)
N^{FF}	30
N^{JJ}	41
N^{FJ}	51
N_{in}^{FF}	31
N_{in}^{JJ}	39
N_{in}^{FJ}	43
N_{out}^{FF}	62
N_{out}^{JJ}	78
N_{out}^{FJ}	72
$N_{a,d}^{FF}$	58
$N_{a,d}^{JJ}$	64
$N_{a,d}^{FJ}$	56
$N_{e,g}^{FF}$	41
$N_{e,g}^{JJ}$	61
$N_{e,g}^{FJ}$	72

Conditions: 50 mM NaCl, 10 mM sodium phosphate (pH 7.0).

the last leucine of the leucine repeat (see Experimental Procedures). As a first step toward identifying sources of stabilization and destabilization in the new Fos and Jun leucine zipper dimers, the pH dependence of the disulfide-bonded peptide pairs was studied (Figure 1A).

The thermal stability of the Fos and Jun homodimers is pH dependent in a dramatic way; the T_m (melting temperature determined from the midpoint of the thermal unfolding transition, see Experimental Procedures) of the Fos homodimer increases ~40°C from neutral to acidic pH, and the stability of the Jun homodimer increases ~20°C from neutral to basic pH (Figure 1A). The stability of the heterodimer changes approximately as expected from an average of the pH dependence of the homodimers.

These large effects of pH on stability can be explained in part by examining the sequences of the Fos and Jun leucine zippers. A particularly useful representation of the sequence is the coiled-coil diagram in which the helices of the dimer are viewed from the N-terminus with the helix axis projecting into the page (Figure 1B). The sequence of coiled-coil proteins can be divided up into positions of the heptad repeat, labeled **a–g** (Hodges et al., 1972; McLachlan and Stewart, 1975). Residues at positions **a** and **d** comprise the 4-3 hydrophobic repeat characteristic of coiled coils, and residues at positions **e** and **g** are predominantly charged amino acids that can be involved in intra- or interhelical electrostatic interactions (Hodges et al., 1972; McLachlan and Stewart, 1975).

The Fos leucine zipper is very acidic, with a high concentration of acidic amino acids at positions **e**, **g**, and **b** (Figure 1B). Because each chain has a large net negative charge at neutral pH (each chain has a net charge of −5), one expects that dimer formation would be disfavored due to general electrostatic repulsion between monomers. Additionally, the alignment of four negatively charged side chains at position **g** along one face of the helix is expected to be a source of intrahelical destabilization. The large increase in stability of the Fos homodimer upon titration to low pH can thus be explained to result from the relief of destabilizing intra- and interhelical electrostatic interactions between acidic residues close to the hydrophobic interface of the dimer.

The Jun leucine zipper has a slight net positive charge at neutral pH (dimer has a net charge of +2 at pH 7); in addition, the concentration of charge in the Jun leucine zipper is more spread out than in Fos (Figure 1B). These properties are consistent with the less dramatic increase in stability of the Jun homodimer at high pH (Figure 1A). Qualitatively, the pH dependence of stability for the Fos–Jun heterodimer changes as expected from an average of the pH dependences for the homodimers. This result suggests that the Fos–Jun leucine zipper lacks dominant stabilizing electrostatic interactions that are unique to the heterodimer; in such a case, a bell-shaped pH dependence curve would be expected. In contrast, the stability of the heterodimer increases at acidic pH values, suggesting that intrahelical repulsion (expected from the Fos sequence) is strong.

Electrostatic effects provide a possible explanation for preferential heterodimer formation in the Fos–Jun system. The data suggest that the peptide homodimers are destabilized at neutral pH by residues of like charge—the Fos homodimer by acidic residues, and the Jun homodimer, to a lesser extent, by basic residues. The interhelical component of this electrostatic destabilization is relieved in the heterodimer because the Fos and Jun monomers are of opposite charge.

The Inside Residues Are Responsible for Specificity and pH-Dependent Stability

To probe the specificity of the Fos–Jun leucine zipper interaction further, structurally based hybrid peptides were made by replacing portions of the Fos and Jun sequences with sequence from GCN4. A peptide corresponding to the leucine zipper region from GCN4 forms very stable homodimers (O'Shea et al., 1989a). As Fos and Jun are likely to fold as coiled coils, the boundary between the Fos or Jun sequence and the GCN4 sequence was set by dividing the helical wheel diagram into two groups of residues: the "inside" group, consisting of the predominantly hydrophobic residues (positions **a** and **d**) and the predominantly charged residues at positions **e** and **g**, and the "outside" group, consisting of residues from positions **b**, **c**, and **f** (Figure 2A).

Two sets of hybrid leucine zipper peptides were constructed. One set of peptides has native sequence (N) from Fos or Jun at the inside positions and outside sequence from GCN4; these peptides are referred to as N_{in} (Figure 2A). The other set of peptides contains GCN4 sequence inside and Fos or Jun sequence outside; these peptides are referred to as N_{out} (Figure 2A).

The preference for heterodimer formation was quantitated from a redox experiment in which an equimolar mixture of the cysteine-containing Fos and Jun peptides is equilibrated in a redox buffer that facilitates disulfide bond formation. K_{redox} is determined from the ratio of disulfide-bonded heterodimer to homodimers (Figure 3). The free

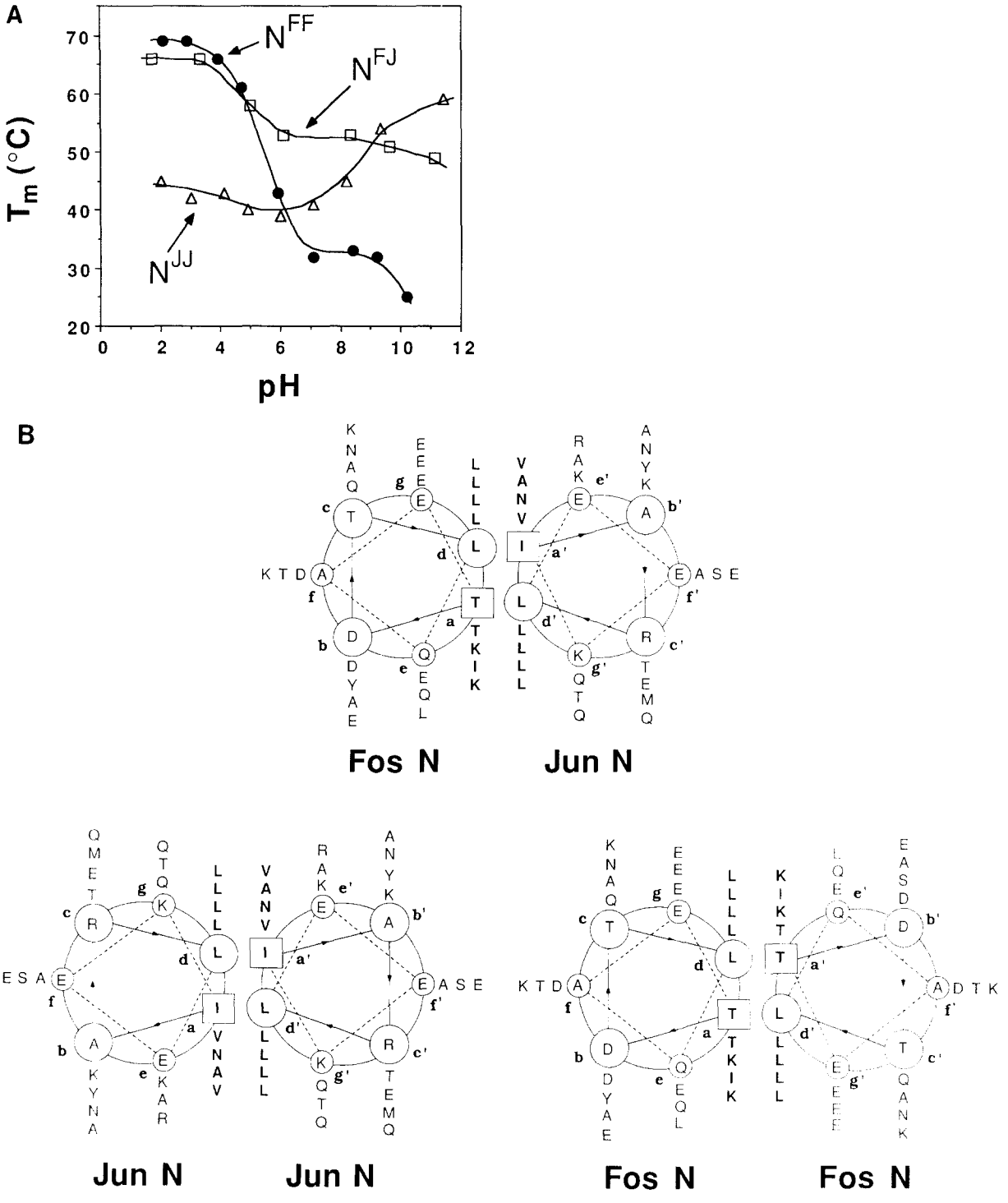


Figure 1. Native Fos and Jun Disulfide-Bonded Leucine Zipper Peptide Dimers

(A) pH dependence of stability for the native Fos peptide dimer (N^{FF}), Jun dimer (N^{JJ}), and heterodimer (N^{FJ}).

(B) Coiled-coil helical wheel representation of the sequences of the Fos N peptide homodimer, Jun N homodimer, and Fos N–Jun N heterodimer. The Fos N peptide homodimer has a net charge of −10 at pH 7, and the Jun homodimer has a net charge of +2.

energy of specificity for heterodimer formation (ΔG_{spec}) is equal to $-RT\ln K_{redox} + RT\ln 2$ (Figure 3). These experiments indicate that there is −2.3 kcal/mol (~100:1) preference for heterodimer in the native peptides (Figure 4).

The N_{in} peptides also form heterodimers preferentially, but with reduced specificity (ΔG_{spec} is −1.2 kcal/mol). The decrease in specificity of the N_{in} peptides appears to arise from a decrease in stability of the N_{in} heterodimer; the N_{in} heterodimer is less stable than the native heterodimer but the stabilities of the N_{in} homodimers are the same as the

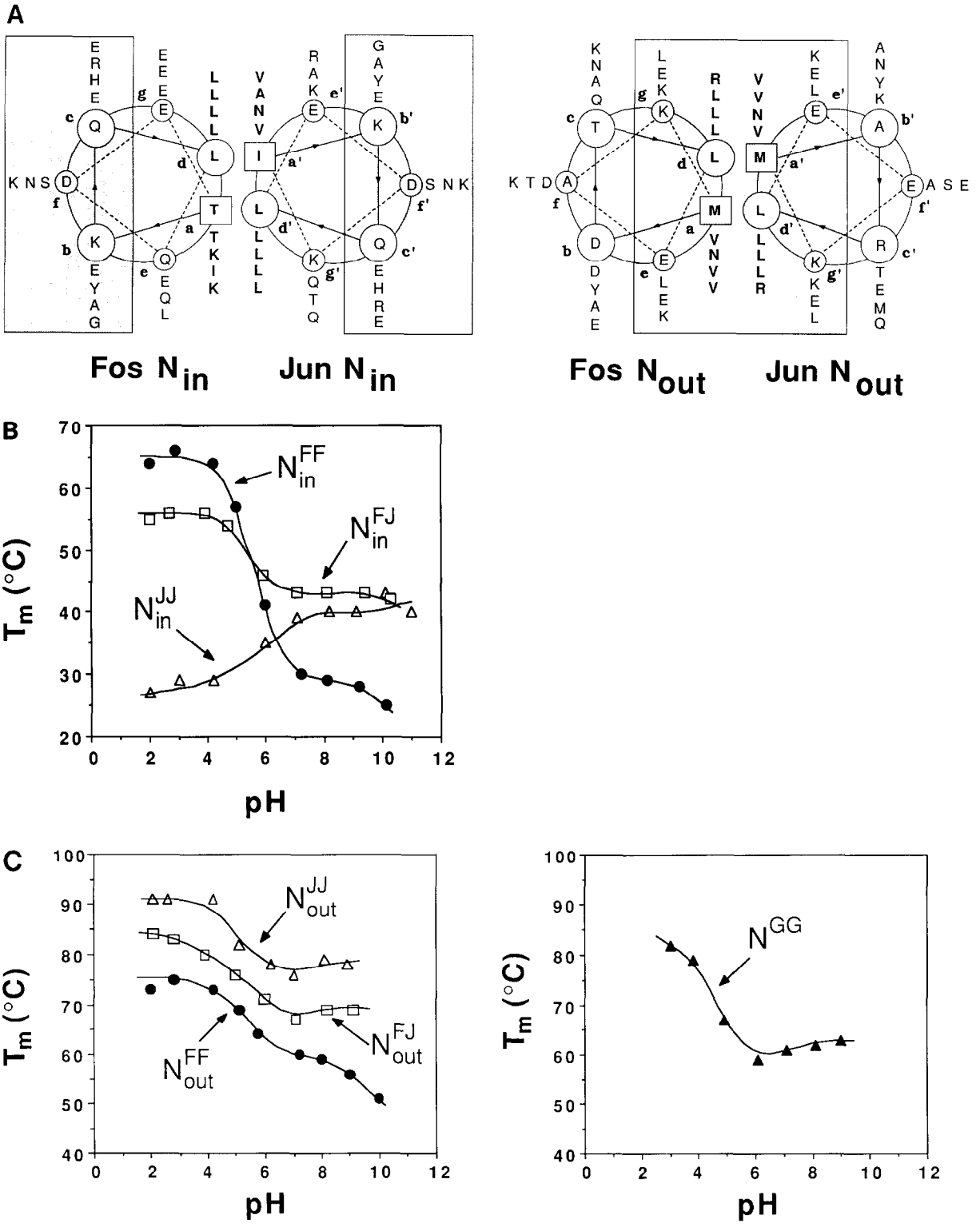
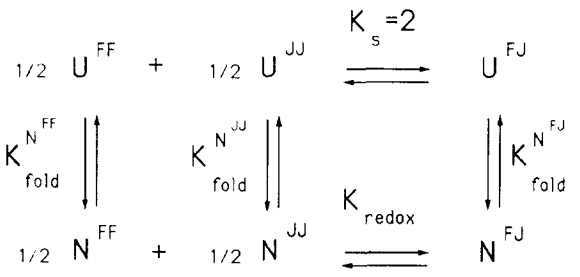


Figure 2. Structurally Based Inside-Outside Hybrid Dimers

(A) Helical wheel representation of the sequence of the N_{in} and N_{out} Fos–Jun heterodimers. Residues within the shaded box are from the GCN4 leucine zipper sequence, and unshaded residues are from the native Fos and Jun leucine zippers.

(B) pH dependence of stability for the N_{in} hybrid hetero- and homodimers. The pH dependence of stability for these hybrid peptides is similar to the pH dependence of stability for the native Fos and Jun peptides, especially for the N_{in} Fos homodimer.

(C) Comparison of the pH dependence of stability for the N_{out} hybrid dimers and the disulfide-bonded GCN4 leucine zipper peptide. The pH dependence of stability for the N_{out} dimers resembles that of the GCN4 leucine zipper dimer.



$$K_{redox} = \frac{[N^{FJ}]}{[N^{FF}]^{1/2}\,[N^{JJ}]^{1/2}}$$

$$\Delta G_{spec} = \Delta G_{redox} + RT\ln 2$$

$$= \Delta G_{fold}(N^{FJ}) - 1/2\,[\Delta G_{fold}(N^{FF}) + \Delta G_{fold}(N^{JJ})]$$

Figure 3. Quantitative Representation of the Preference for Heterodimers

A thermodynamic cycle describing equilibria between the disulfide-bonded Fos and Jun peptide dimers is shown. U represents the unfolded state; N is the folded state; FF, JJ, and FJ are the disulfide-bonded Fos–Fos, Jun–Jun, and Fos–Jun peptide dimers, respectively. K_{redox} is the equilibrium constant measured by redox experiments, as described in the Experimental Procedures. K_s represents the equilibrium constant for random pairing of Fos and Jun peptides; the value of K_s is 2 because there are two ways to form the heterodimer and only one way to form each homodimer. K_{fold} is the folding equilibrium constant (for the disulfide-bonded peptide pairs). ΔG_{spec} is a quantitation of specificity and can be expressed in terms of either K_{redox} or in terms of K_{fold} for each of the disulfide-bonded dimers.

corresponding native homodimers (Table 1). In contrast, the N_{out} peptides show essentially no specificity (ΔG_{spec} is −0.1 kcal/mol). As expected if there is no preference for heterodimer over homodimers, the N_{out} heterodimer has a stability that is intermediate between that of the two N_{out} homodimers (Table 1). Therefore, the inside residues of the Fos and Jun leucine zipper are necessary and sufficient to mediate preferential heterodimer formation.

The N_{in} peptides show pH-dependent stability that closely resembles that of the native Fos and Jun peptides (Figure 2B). In contrast, the pH dependence for each of the N_{out} dimers does not resemble that of the corresponding native dimer, but resembles that of the GCN4 leucine zipper peptide (Figure 2C). Therefore, residues at the inside positions (positions **a**, **d**, **e**, and **g**) are also largely responsible for the pH-dependent stability observed with the Fos and Jun peptide dimers.

Sequence Requirements for Specificity

To investigate more thoroughly the sequence requirements for specificity, all possible combinations of the previously described seven peptides were made (there are 28 possible combinations, and 21 of these are heterodimers), and the stability of each disulfide-bonded dimer was measured by thermal denaturation (Figure 5A). There are large differences in stability; the T_ms range from 17°C–83°C. The free energy of specificity can be approximated by the difference in T_m between a given heterodimer and the average of the T_ms for the corresponding homodimers (ΔT_m = T_m(heterodimer AB) − 1/2 [T_m(homodimer AA) + T_m(homodimer BB)]). For the Fos–Jun peptides (see Figure 4), ΔT_m appears to be a quantitative measure of specificity, as it is linearly related to the free energy of specificity, ΔG_{spec}, with a proportionality constant of 7.4°C/kcal.

Using ΔT_m as a measure of specificity, the heterodimeric peptide pairs can be grouped into three classes (Figure 5B): specificity, antispecificity, and additive. The specificity class includes peptide pairs with positive, nonadditive differences in T_m ($\Delta T_m \geq +8$°C); the antispecificity class contains peptide pairs with negative, nonadditive differ-

		ΔG_{spec} (kcal/mol)	ΔT_m (°C)
Jun N + Fos N	+	-2.3 ± 0.09	+16
Jun N_{in} + Fos N_{in}	+	-1.2 ± 0.03	+8
Jun N_{out} + Fos N_{out}	+	-0.1 ± 0.03	+2
Jun $N_{a,d}$ + Fos $N_{a,d}$	+	+0.5 ± 0.04	-5
Jun $N_{e,g}$ + Fos $N_{e,g}$	+	-2.8 ± 0.2	+21

Figure 4. Values for ΔG_{spec} and ΔT_m for Various Peptide Combinations

ΔG_{spec} and ΔT_m were determined as described in the text. The reported value of ΔG_{spec} is the mean of at least six redox experiments, and the error reported is ± one standard deviation.

$$\Delta G_{spec} = -RT\ln K_{redox} + RT\ln 2$$

$$\Delta T_m = T_m \text{ (heterodimer AB) - 1/2 [} T_m \text{ (homodimer AA) + } T_m \text{ (homodimer BB)]}$$

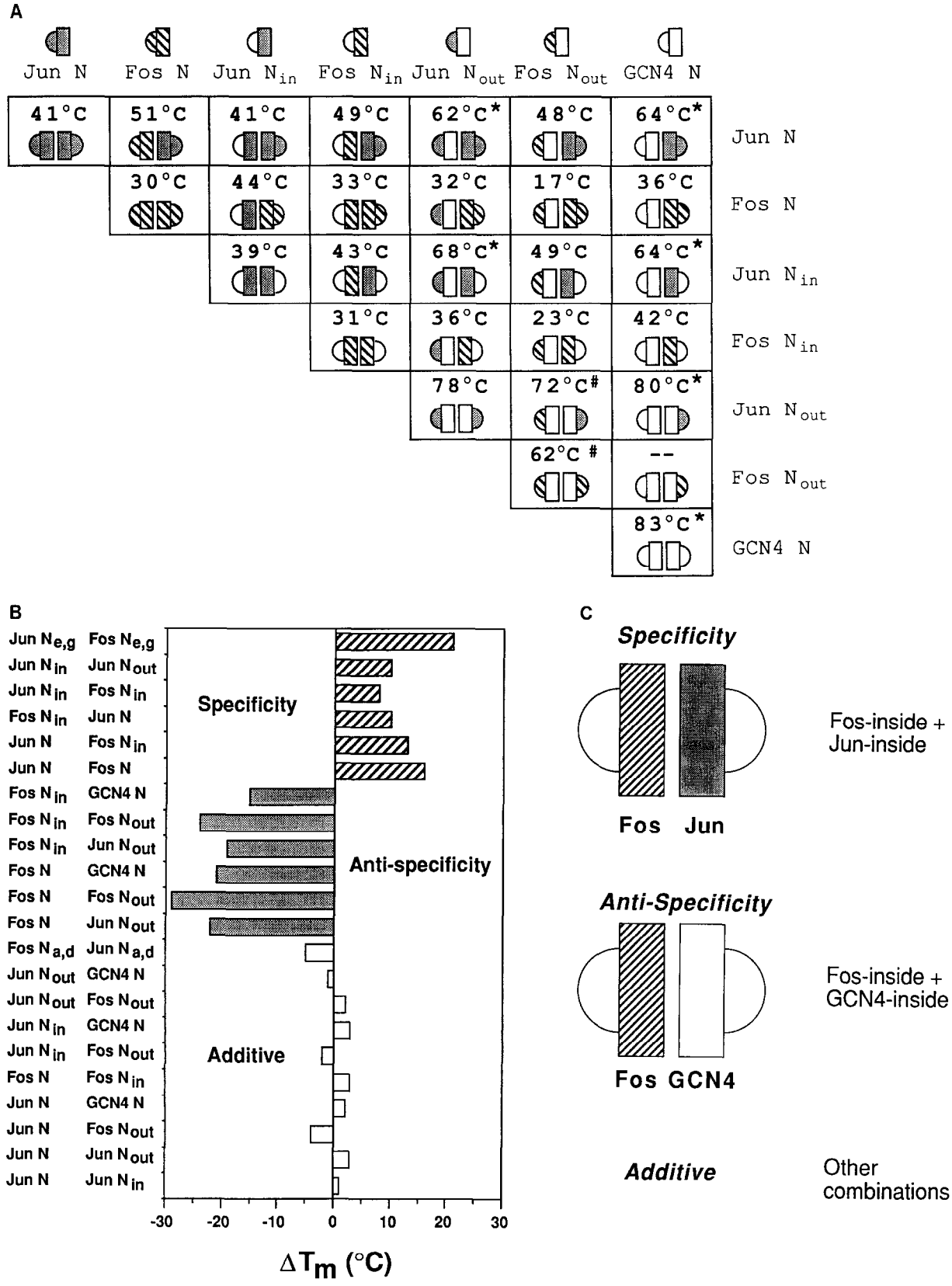


Figure 5. Sequence Determinants of Specificity

(A) Matrix of T_ms for 28 disulfide-bonded dimers. Melting temperatures reported are those determined by curve fitting (see Experimental Procedures). Greater than 75% of the folded CD signal is recovered in all melting curves upon cooling, with the exception of those dimers marked with an asterisk. The peptides marked with an asterisk have undergone some degradation and/or modification during the melting curve determination, as judged by reverse-phase HPLC. Those dimers marked with a number sign have a T_m that is slightly dependent upon peptide concentration, suggesting

ences in T_m ($\Delta T_m \leq -8°C$); and the additive class consists of peptide pairs in which the stability of the heterodimer is intermediate between that of the homodimers ($+8°C > \Delta T_m > -8°C$).

All peptide dimers combining Jun sequence inside with Fos sequence inside fall into the specificity class (Figure 5C). Although the mechanism of antispecificity is not apparent at this time, all members of the antispecificity class have Fos sequence inside combined with GCN4 sequence inside. The other peptide combinations fall into the additive class, with one exception (the Jun N_{in}–Jun N_{out} heterodimer falls into the specificity class, for reasons that are not readily apparent). The most striking result is that specific heterodimer formation is observed with all peptide pairs containing Fos sequence inside combined with Jun sequence inside (Figure 5), regardless of the sequences at the outside positions, reinforcing the previous conclusion that the inside residues (positions **a**, **d**, **e**, and **g**) are the major determinant of peptide pairing.

The inside residues consist of the predominantly hydrophobic positions (**a** and **d**) and predominantly charged positions (**e** and **g**). GCN4-based hybrid peptides containing native Fos or Jun sequence at the hydrophobic positions ($N_{a,d}$) or the predominantly charged positions ($N_{e,g}$) were made to evaluate the contribution of these groups of residues to specificity (Figure 6A). The $N_{e,g}$ peptides form heterodimers with specificity (ΔT_m and ΔG_{spec}) at least as great as that of the native sequences (Table 1; Figure 4). In contrast, the $N_{a,d}$ peptides are slightly antispecific.

Thus, 8 residues at positions **e** and **g** of Fos and Jun are sufficient to mediate preferential heterodimer formation. Although the residues from Fos and Jun that comprise the hydrophobic interface between the helices (positions **a** and **d**) are undoubtably important for stability (Smeal et al., 1989; Ransone and Verma, 1990), these residues do not appear to be important for specificity. We conclude that van der Waals packing differences do not have a dominant role in the discrimination between the Fos–Jun heterodimer and the corresponding homodimers. Rather, the mechanism of specific heterodimer formation appears to be predominantly electrostatic in nature.

This conclusion is supported further by the finding that the $N_{e,g}$ hybrids show pH-dependent stability similar to that observed with the native peptides (Figure 6B). In particular, the Fos $N_{e,g}$ homodimer exhibits very strong pH-dependent stability (T_m = 41°C at pH 7 and >90°C at pH 4). In contrast, the pH dependence of the $N_{a,d}$ hybrid dimers does not resemble that of the native peptides (Figure 6C). Thus, residues at positions **e** and **g** in the Fos and Jun sequences also account in large part for the dramatic pH-dependent stabilities observed with the native peptides.

Discussion

The requirements for specificity in the Fos–Jun system appear to be simple: 8 residues from Fos and from Jun, in a background of the GCN4 leucine zipper, are sufficient to mediate preferential heterodimer formation. pH dependence studies suggest a mechanism for specificity in which destabilization of the Fos homodimer by acidic residues (at positions **e** and **g**) shifts the dimerization equilibrium toward the Fos–Jun heterodimer. Therefore, preferential heterodimer formation by the Fos and Jun leucine zipper peptides is largely a thermodynamic consequence of Fos homodimer instability (O'Shea et al., 1989b). Destabilization of a homodimer is also used to provide specificity in the case of the tropomyosin αβ heterodimer (Lehrer et al., 1989; Lehrer and Stafford, 1991).

The coupling of the ionization state of residues at positions **e** and **g** to the stability of the Fos and Jun leucine zippers can be rationalized by using the crystal structure of a peptide corresponding to the GCN4 leucine zipper (O'Shea et al., 1991). In this crystal structure, the methylene groups of the predominantly charged residues at positions **e** and **g** pack against the predominantly hydrophobic residues at positions **a** and **d** (Figure 7). Thus, the hydrophobic interface is actually formed by side chains from 4 residues of the heptad repeat. Additionally, terminal charged groups of residues at positions **e** and **g'** of the preceding heptad are close to each other. It is likely that the close proximity of negatively charged residues at positions **e** and **g** of opposing Fos monomers would disrupt the complementary packing seen at the dimer interface of the coiled coil, accounting for the instability of the Fos homodimer at neutral pH.

Studies of the requirements for specificity in the Fos and Jun proteins reinforce the conclusions drawn from the peptide studies described here. Schuermann et al. (1991) have systematically substituted residues from each position of the heptad repeat of Jun (**a**, **b**, **c**, **e**, **f**, and **g**) into the Fos leucine zipper and assayed for preferential heterodimer formation by coimmunoprecipitation. Although these authors reach a slightly different conclusion, their results demonstrate that only substitutions at positions **e** and **g** affect substantially the amount of heterodimer obtained.

The agreement between the studies of leucine zipper

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that they may be associating into higher order oligomers (see Experimental Procedures). The Jun N_{out}–GCN4 N heterodimer has a melting curve with two overlapping transitions, so no T_m is reported for this dimer. This peptide appears to be modified and/or degraded during the thermal melt, as judged by HPLC.

(B) Plot of ΔT_m for the disulfide-bonded heterodimers. ΔT_m is the difference in T_m between a given heterodimer and the average T_m for the corresponding homodimers. The heterodimers can be grouped into three classes based on ΔT_ms: ΔT_ms $\geq +8°C$ are in the specificity class; ΔT_ms $\leq -8°C$ are in the antispecificity class; and $+8°C > \Delta T_m$s $> -8°C$ are in the additive class. ΔT_m is linearly proportional to the free energy of specificity, ΔG_{spec}, derived from redox experiments (see Figure 4).

(C) Dimers that belong to each ΔT_m class. All peptides with Fos inside (positions **a**, **d**, **e**, and **g**) combined with Jun sequence inside belong to the specificity class. The only other member of the specificity class is Jun N_{in}–Jun N_{out}. All peptides with Fos sequence inside combined with GCN4 sequence inside belong to the antispecificity class. Other combinations of peptides belong to the additive class.

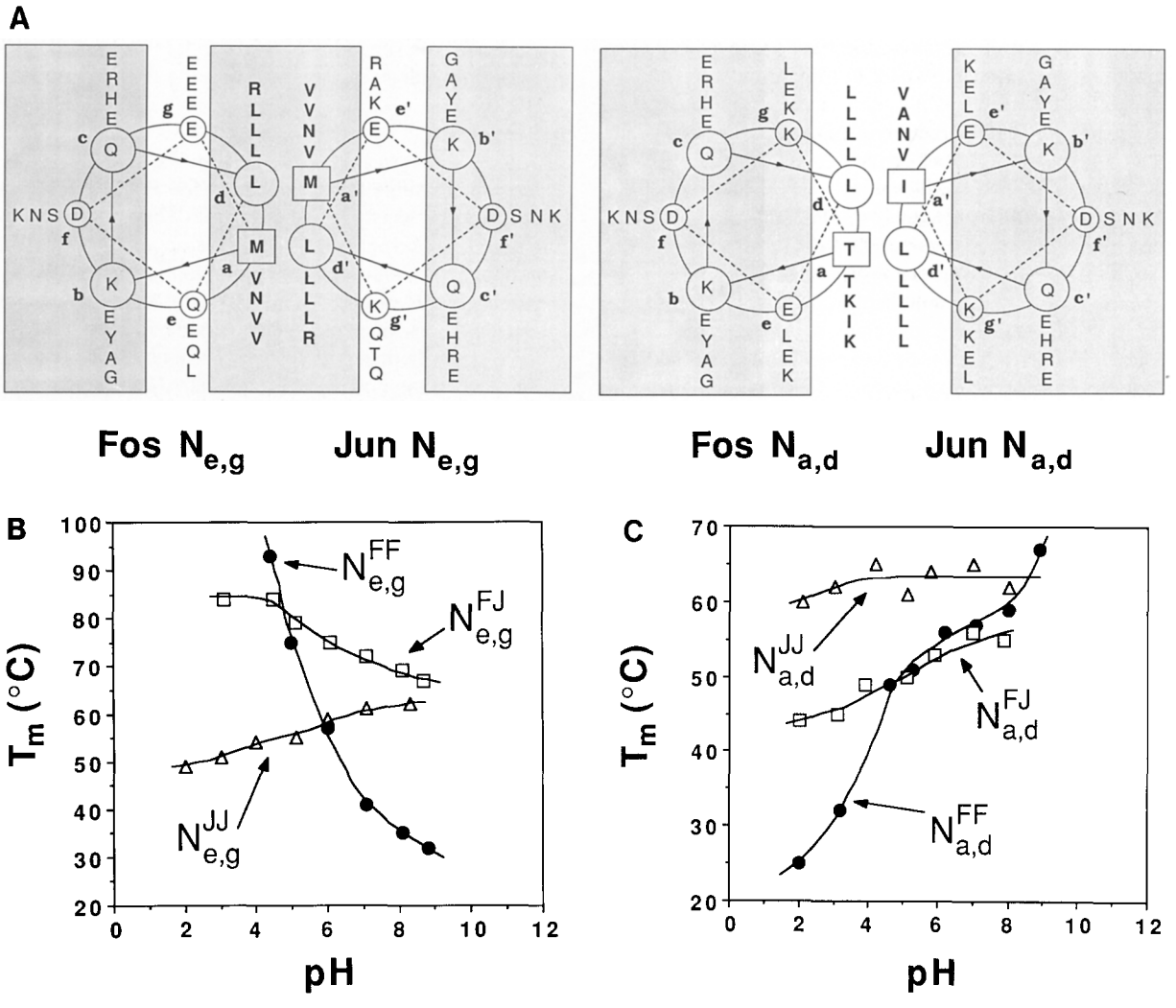

Figure 6. $N_{a,d}$ and $N_{e,g}$ Hybrid Dimers

(A) Helical wheel representation of the sequence of the $N_{a,d}$ and $N_{e,g}$ Fos–Jun heterodimers. Residues within the shaded box are from the GCN4 leucine zipper sequence, and unshaded residues are from the native Fos and Jun leucine zippers.

(B) The pH dependence of stability for the hybrid $N_{e,g}$ dimers resembles the pH dependence of stability for the native Fos and Jun leucine zipper peptides (Figure 1A).

(C) The pH dependence of stability for the hybrid $N_{a,d}$ dimers does not resemble that of the native Fos and Jun peptides (Figure 1A).

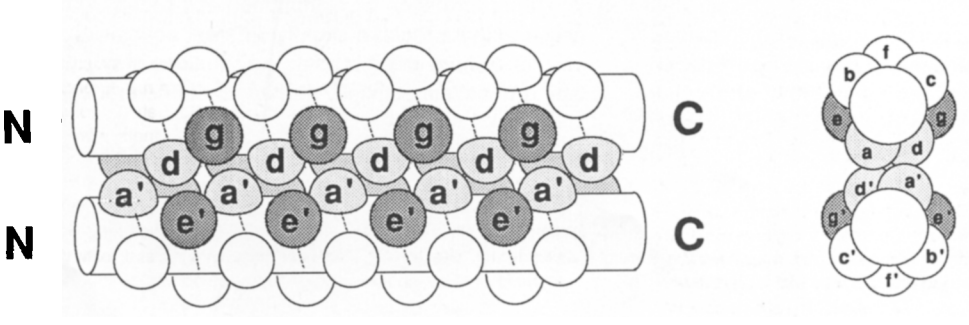

Figure 7. Side and End Views of a Coiled Coil Illustrating Interactions Seen in the Structure of the GCN4 Leucine Zipper

For simplicity, the supercoiling of the helices is not depicted. Prime refers to the opposing helix. The dimer interface is composed of residues from the 4-3 hydrophobic repeat (positions **a** and **d**), as well as the predominantly charged residues (positions **e** and **g**). Methylene groups in the side chains of residues at positions **e** and **g** pack against residues at positions **a** and **d**. Additionally, the terminal charged groups of residues at position **e′** and position **g** of the preceding heptad repeat are seen to be close to one another in the crystal structure of a peptide corresponding to the GCN4 leucine zipper (O'Shea et al., 1991).

peptides and the Fos and Jun proteins suggests that the conclusions derived from studies of isolated leucine zipper peptides are applicable to the intact proteins. The ability to study small pieces of proteins simplifies greatly many problems of protein structure and stability. It is likely that this peptide approach will be useful in studying homo- and heterotypic interactions between other transcription factor domains. Principles of specificity of the type learned from the Fos–Jun peptide system should assist in the design and prediction of coiled-coil sequences that preferentially form homo- or heterodimers.

Experimental Procedures

Peptide Synthesis and Purification

Peptides were synthesized using t-BOC chemistry on an Applied Biosystems model 430A peptide synthesizer with standard reaction cycles modified to include acetic anhydride capping (for a review, see Kent, 1988). Peptide Jun N corresponds to residues 286–317 of the c-Jun protein (Bohmann et al., 1987; Maki et al., 1987), and peptide Fos N corresponds to residues 162–193 of the c-Fos protein (Van Beveren et al., 1983; van Straaten et al., 1983). Ser-295 of c-Jun and Ser-177 of c-Fos have been replaced with tyrosine to facilitate concentration determination by UV absorbance measurements. Peptide GCN4 N consists of residues 250–281 of the GCN4 protein (Hinnebusch, 1984). All peptides have the sequence Cys-Gly-Gly appended to the N-terminus, are acetylated at the N-terminus, and are amidated at the C-terminus. Peptides were cleaved by either low/high HF cleavage (Immunodynamics, Inc., San Diego, CA) or by trifluoromethanesulfonic acid cleavage (Kent, 1988) and were desalted on a Sephadex G-10 column (Pharmacia) in 5% acetic acid. Final purification was by reverse-phase high performance liquid chromatography (HPLC) (Waters, Inc. and Applied Biosystems) using a Vydac preparative C18 column (2.2 × 25 cm) at 25°C or 50°C. A linear acetonitrile–H_2O gradient with segments of 0.1% to 0.2% buffer B increase per minute was used with a flow rate of 20 ml/min. Buffer A consisted of water with 0.1% trifluoroacetic acid, and buffer B consisted of 90% acetonitrile, 10% water with 0.1% trifluoracetic acid. The identity of each peptide was confirmed by fast atom bombardment mass spectrometry (M-Scan, Inc., West Chester, PA or Mass Search, Inc., Modesto, CA) and was found to be within 1 dalton of the expected mass.

Circular Dichroism Studies

Circular dichroism (CD) studies were performed using a 1 cm or 1 mm cuvette (Helma or Uvonic) on an Aviv CD spectrophotometer (model 60DS or model 62DS) equipped with a thermoelectric controller. The buffer used for all CD experiments except pH titrations was 50 mM NaCl, 10 mM sodium phosphate (pH 7.0). All peptide concentrations were determined by tyrosine absorbance (Edelhoch, 1967) at 275.5 nm in 5.4–6 M GuHCl (Schwarz/Mann Biotech Ultra-Pure grade) using an Aviv UV/VIS spectrophotometer (model 18DS or 14DS). The molar ellipticity at 222 nm, 0°C, was measured for the Fos and Jun N, N_{in}, N_{out}, $N_{a,d}$, and $N_{e,g}$ disulfide-bonded homo- and heterodimers and for the GCN4 N homodimer. All values were found to be within the range −27,000 to −33,000 deg cm^2 dmol^{-1}, indicating that the peptides are >80% helical. Thermal melting curves were determined by monitoring the CD signal at 222 nm as a function of temperature. The pH dependence of stability was measured in 50 mM NaCl, 10 mM sodium phosphate at various pH values. Because the disulfide-bonded GCN4 N dimer is very stable, 2 M GuHCl (Schwarz/Mann Biotech Ultra-Pure grade) was included in the buffer used for monitoring the pH dependence of stability for this peptide. The same overall shape of the pH dependence curve is obtained in the absence of GuHCl. Reversibility was checked for all thermal melts. In general, melting curves obtained above pH 8 are not reversible, presumably because of chemical modification and/or degradation (observed by HPLC). Disulfide-bonded dimers that have irreversible melting curves (<75% of folded signal is recovered upon cooling) at pH 7.0 are marked (asterisk) in Figure 5A. These peptides have undergone chemical modification and/or degradation during the thermal melt, as judged by subsequent HPLC analysis.

Determination of the T_m

The T_m was determined by curve fitting the thermal denaturation curve to the following equation using a nonlinear least squares–fitting program (Kaleidagraph, Synergy Software):

$$\theta = \theta_F(0\text{ K}) + m_F T + [\theta_U(0\text{ K}) + m_U T - \theta_F(0\text{ K}) - m_F T]\,[e^{(\Delta H/RT + \Delta S/R)}/1 + e^{(\Delta H/RT + \Delta S/R)}]$$

where T is temperature in K; θ is the CD signal at 222 nm; θ_F (0 K) is the value for the CD signal of the folded peptide extrapolated linearly to 0 K; m_F is the slope of the temperature dependence of the CD signal for the folded peptide; m_U is the slope of the temperature dependence of the CD signal for the unfolded peptide; θ_U (0 K) is the value for the CD signal of the unfolded peptide extrapolated linearly to 0 K; ΔH is the enthalpy of unfolding at the midpoint of the thermal denaturation curve; and ΔS is the entropy of unfolding at the midpoint of the thermal denaturation curve. The T_m is the temperature at which the fraction unfolded is equal to the fraction folded ($\Delta G = 0$), and it is equal to $\Delta H/\Delta S$. The assumptions that were made in using the equation above are as follows: thermal melting curves are two state, described by an equilibrium between unfolded and folded peptide; and the enthalpy and entropy of unfolding are independent of temperature.

Because some of the Fos peptide homodimers and Fos N_m–GCN4 N_m peptide heterodimers are not completely folded at 0°C, the slope of the folded baseline (m_F) and the value for the CD signal of the folded baseline extrapolated to 0 K (θ_F (0 K)) for each of these dimers were determined from a melting curve at pH 2.0 (conditions where the Fos-containing peptides are more stable).

For each peptide the T_m was also determined by taking the first derivative of the CD signal (θ) with respect to temperature^{-1} (temperature in K) and finding the minimum of this function (Cantor and Schimmel, 1980). All reported values of T_m are those determined from curve fitting. The error in the measurement of T_m (estimated from the width of the $d\theta/d(1/T)$ plot and from repeated curve fits starting from independent points) is ±2°C except in cases in which 20°C > T_m > 80°C, where the error is ±5°C. The determinations of T_m by $d\theta/d(1/T)$ and by curve fitting agree to within the estimated errors. Additionally, the T_m for each disulfide-bonded dimer was measured as a function of peptide concentration (over at least a 2.5-fold range of peptide concentration in the low micromolar range, as estimated by the ratio of the CD signal at low and high concentration) to determine if the dimers were associating to higher order oligomers (O'Shea et al., 1989a, 1989b). Peptide dimers that were found to have a T_m that is dependent upon peptide concentration (~3°C change in T_m over a 3- to 4-fold concentration range) are marked in Figure 5A (number sign), and the T_m reported is the higher of the two measurements.

Redox Experiments

The disulfide-bonded heterodimer was incubated in redox buffer consisting of 100–500 μM reduced glutathione (Sigma), 100–500 μM oxidized glutathione (Sigma), 50 mM NaCl, 10 mM sodium phosphate (pH 7.4) at ~23°C in an anaerobic chamber (Coy Laboratory Products, Inc.). Reactions were equilibrated at a total peptide concentration of ~10–50 μM for 6–16 hr and quenched under anaerobic conditions with concentrated formic acid to a final concentration of 5% by volume (pH < 2). The reaction products were analyzed by microbore HPLC (Waters, Inc.) using a linear acetonitrile–H_2O gradient with segments of 0.1% to 0.25% increase in buffer B per minute. An analytical Vydac C-18 column (0.46 × 25 cm) was used at a column temperature of 25°C, 40°C, or 50°C with a flow rate of 0.2 ml/min. Relative concentrations of the disulfide-bonded hetero- and homodimers were determined by integration of the corresponding peaks (absorbance at 229 nm was monitored). Each redox reaction was determined to be at equilibrium by repeating the reaction using an equimolar mixture of reduced peptides as the starting material. The values for ΔG_{spec} obtained from these two different starting points agreed to within 0.1 kcal/mol.

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