

Improved Stability of the Jun-Fos Activator Protein-1 Coiled Coil Motif

A STOPPED-FLOW CIRCULAR DICHROISM KINETIC ANALYSIS*

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Two c-Jun leucine zipper variants that bind with high affinity to c-Fos have been selected using semirational design combined with protein-fragment complementation assays (JunW) or phage display selection (JunW_{Ph1}). Enriched winners differ from each other in only two of ten semi-randomized positions, with ΔT_m values of 28 °C and 37 °C over wild-type. cFos-JunW, cFos-JunW_{Ph1}, and two intermediate mutants (cFos-JunW_{Q21R} and cFos-JunW_{E23K}) display biphasic kinetics in the folding direction, indicating the existence of a folding intermediate. The first reaction phase is fast and concentration-dependent, showing that the intermediate is readily populated and dimeric. The second phase is independent of concentration and is exponential. In contrast, in the unfolding direction, all molecules display two-state kinetics. Collectively this implies a transition state between unfolded helices and dimeric intermediate that is readily traversed in both directions. We demonstrate that the added stability of cFos-JunW_{Ph1} relative to cFos-JunW is achieved via a combination of kinetic rate changes; cFos-JunW_{E23K} has an increased initial dimerization rate, prior to the major transition state barrier while cFos-JunW_{Q21R} displays a decreased unfolding rate. The former implies that improved hydrophobic burial and helix-stabilizing mutations exert their effect on the initial, rapid, monomer-collision event. In contrast, electrostatic interactions exert their effect late in the folding pathway. Although our focus is the leucine zipper region of the oncogenic transcription factor Activator Protein-1, coiled coils are ubiquitous and highly specific in their recognition of partners. Consequently, generating kinetic-based rules to predict and engineer their stability is of major significance in peptide-based drug design and nano-biotechnology.

The required search through conformational space is not a random one, as was first highlighted by Cyrus Levinthal (1). Rather, protein chains are guided both thermodynamically and kinetically to the point of lowest free energy; usually the folded state (2). Much progress has been made in this field, in large by studying small monomeric proteins (3–5) where often only the unfolded and folded states are significantly populated (6, 7). However, studying proteins that contain quaternary structure is of great appeal and is certainly more useful in the design of peptide based drugs, presenting the possibility to engineer protein stability at the protein-protein interface in addition to elucidating the overall folding mechanism. However, studying either proteins with quaternary structure, or proteins that are large (>100 residues) leads to complications in the folding pathway; e.g. multiple transition states, intermediates, and even multiple pathways, making it extremely difficult to extract useful mechanistic information. However, a number of small dimeric proteins have been successfully studied owing to more straightforward two- or three-state folding mechanisms (8–12).

Of these proteins, the parallel dimeric coiled coil appears to be one of the best understood (11–16), as this protein contains only α -helical elements of secondary structure. Indeed, a wealth of reports have been published on the folding of the yeast transcription factor GCN4-p1 (12, 14, 17–21) with many reporting the protein to fold in a two-state mechanism. However, some have speculated about the existence of intermediates (22–24). The mammalian homologues of GCN4, namely c-Jun and c-Fos, dimerize to form the oncogenic transcription factor Activator Protein-1 (AP-1).³ This protein plays a central role in the cell signaling cascade, and is a pivotal transcription factor involved in proliferation and oncogenesis (25, 26). AP-1 therefore presents an interesting, highly relevant, and yet poorly understood model in the study of folding and binding, but also in designing new dominant negative variants with improved K_D over the wild-type; the ultimate aim being to specifically inhibit oncogenic proteins in cancerous situations.

A problem that emerges from studying these oligomeric proteins, however, is that folding and binding is concomitant (12, 27), and that folding is also dependent on the concentration of monomer. This in turn has implications on how the folding mechanism should be studied; some have monitored folding using tryptophan fluorescence (17), or using fluorescent dyes

Understanding how a specific sequence of amino acids is able to form a unique and functional three-dimensional structure has long been regarded as the last piece of the genetic jigsaw.

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³ The abbreviations used are: AP-1, activator protein-1; CD, circular dichroism; GuHCl, guanidine hydrochloride; PCA, protein-fragment complementation assay; F_U, fraction unfolded.

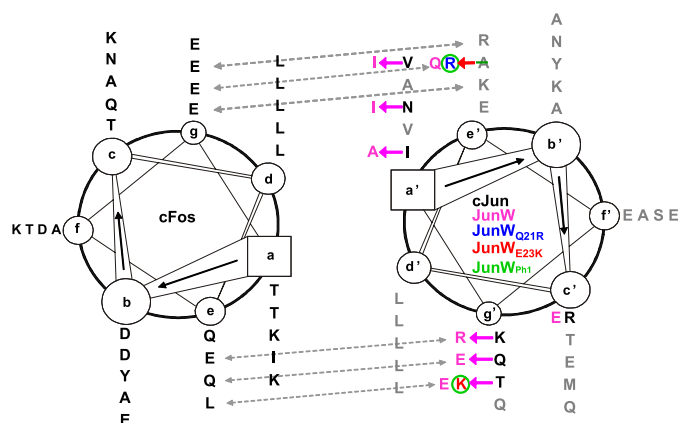


FIGURE 1. Helical wheel representations of the AP-1 Jun-Fos coiled coil, looking down the helical axis in the N- to C-terminal direction. The c-Fos helix (left) was not varied. The Jun-based helix (right) shows residue variations for the five helices. In gray are the residues common to all Jun variants. Black indicates those residues unique to c-Jun. Magenta indicates residues found in JunW variants. An Arg (blue) at position 21 (e'3) represents the Q21R mutation. Likewise, a Lys (red) at position 23 (g'3) represents the E23K mutation. A green circle around both of these residues highlights the double mutant JunW_{Phi1}.

tagged to the termini (14), while others have opted for a global measure of helicity using a stopped-flow circular dichroism technique (12). This last method appears to be the more informative, since fluorescence changes in solvent exposed tryptophan residues (where fewer structural perturbations are imposed) or in terminal dyes would inform poorly on early helix formation events prior to monomer collision. This is because changes in solvation are unlikely to be large in such early folding events. Some have circumvented this problem by disulfide-bridging one monomer to another and monitoring helicity as a function of time (19, 21, 28), effectively removing both problems. However, the validity of this is questionable when extrapolating the information back to the wild-type dimeric protein, and has been speculated to attain the downhill folding limit by simply “zipping up” without a folding barrier (28). Nonetheless, additional identifiable steps on the folding pathway, despite the added complexities, permit more information to be extracted regarding the level of structure and hydrophobic exposure along the folding coordinate.

We previously constructed high affinity peptide variants directed at the leucine zipper regions of c-Jun and c-Fos (see Fig. 1) (29).⁴ The ultimate aim of this study was to create dominant negatives that bind to either Fos or Jun with much improved stability over wild-type, and to understand how sequence defines structure. Extracting information on how key residues at crucial positions within the structure confer kinetic rules for stability would permit fast tracking the design process for future variants. We therefore explored how differences in stability for these variants are manifested. Is stability achieved at the kinetic level where dimeric proteins are able to fold faster, because of a lowering of the transition state barrier, relative to the unfolded protein? Conversely, is the stability achieved at the thermodynamic level, by raising the height of the transition state barrier relative to the folded state? Answering these ques-

tions will permit designing for improved stability protein-protein interactions, which fulfill these criteria from the outset.

EXPERIMENTAL PROCEDURES

Peptide Purification—Peptides (cFos: ASTDTLQAETDQL-EDEKYALQTEIANLLKEKEKLGAP; cJun: ASIARLEEKVKTLKAQNYELASTANMLREQVAQLGAP; JunW: ASAAELEE-RVKTLKAEIYELQSEANMLREQIAQLGAP; JunW_{Q21R}: ASAAELEEERVKTLKAEIYELRSEANMLREQIAQLGAP; JunW_{E23K}: ASAAELEEERVKTLKAEIYELQSKANMLREQIAQLGAP; JunW_{Phi1}: ASAAELEEERVKTLKAEIYELRSKANMLREQIAQLGAP) were synthesized by Protein Peptide Research Ltd (Fareham, UK) and subsequently purified to >98% purity using RP-HPLC with a Jupiter Proteo Column (4- μ m particle size, 90-Å pore size, 250 $\times$ 10 mm; Phenomenex) and a gradient of 5–60% acetonitrile (0.1% trifluoroacetic acid) at 1.5%/min. Residue changes relative to JunW are highlighted in bold. Masses corresponding to the major peaks were assessed using in-house electrospray mass spectrometry. Peptide concentrations were determined in water by absorbance at 280 nm with an extinction of 1209 M⁻¹ cm⁻¹ (31) corresponding to a tyrosine residue inserted into a solvent-exposed b3 position.

Equilibrium Denaturation—Circular dichroism spectra and chemical denaturation profiles were performed at 20 μ M total peptide concentration in 10 mM potassium phosphate, 100 mM potassium fluoride, pH 7.0. Ultra-pure guanidine hydrochloride (GuHCl) was purchased from Carl Roth Chemicals GmbH (Karlsruhe, Germany) and concentrated to 6 M as determined by refractometry. The CD signal at 222 nm, corresponding to the α -helical content, was allowed to equilibrate before being recorded using a 1-cm pathlength CD cell in a Jasco J810 Circular Dichroism spectrophotometer. Sequential 50- μ l additions of 20 μ M total peptide (in 10 mM potassium phosphate, 100 mM potassium fluoride, 6 M GuHCl, pH 7.0) were added and the CD signal recorded, following equilibration, to ensure that the protein concentration in the CD cell remained constant. All reaction solutions were maintained at 293 K using a thermostatted circulating water bath and were monitored continuously with a sensitive thermocouple. From this, the fluctuation in temperature was determined to be no more than (0.1 K). Thorough mixing of the solutions was ensured using a magnetic bar.

Stopped-flow Circular Dichroism (SF-CD)—Folding measurements (Fig. 2A) were initiated by mixing a 220 μ M solution of unfolded peptide containing 10 mM potassium phosphate, 100 mM potassium fluoride, and 3.5 M GuHCl (pH 7.0) against 10 volumes of the given concentration of GuHCl at 293 K in a PiStar-180 (π^* -180) SF-CD apparatus (Applied Photophysics Ltd) to give a postmix peptide concentration of 20 μ M. In the case of the wild-type protein, cFos-cJun, a 40 μ M post-mix total peptide concentration at 283 K was still not sufficient to be able to monitor folding or unfolding. For all other samples, k_{f1} was calculated from k_{app} according to Equation 12. The relationship between the first phase and protein concentration was found to be linear within the 5–20 μ M range (Fig. 3A). A wavelength of 222 nm was selected using entrance and exit slit widths of 4 mm. The post-mix concentration of GuHCl was calculated according to the following: (3.5 M + (pre-mix [GuHCl] $\times$ 10))/

⁴ U. B. Hagemann, J. M. Mason, K. M. Müller, and K. M. Arndt, submitted manuscript.

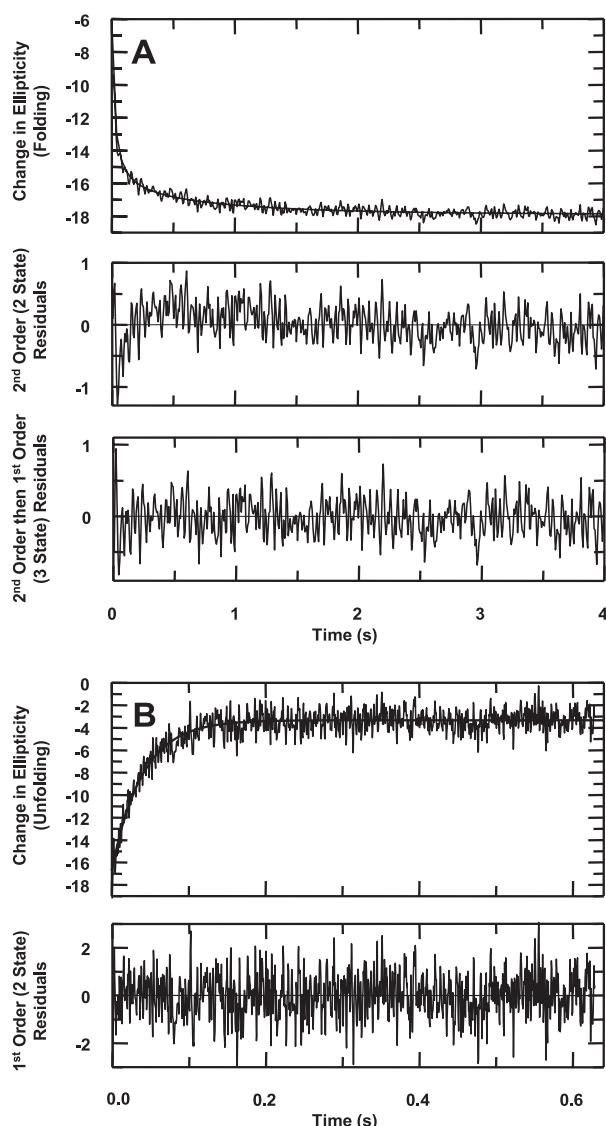


FIGURE 2. **Folding and unfolding behavior of the cFos-JunW_{E23K} variant (pH 7, 293 K, 20 μ M).** A, solid lines represent the three-state fit (Equation 11) to cFos-JunW_{E23K} folding data (3.5 M $\rightarrow$ 0.49 M GuHCl dilution), shown below are the residuals for two-state and three-state fits to the data. Only the latter is a satisfactory fit. B, single exponential fit (Equation 13) to unfolding data (0 M $\rightarrow$ 3.13 M GuHCl), below are the residuals for this fit. In both reactions the earliest measurable signal is equal to the value for the initial state measured separately indicating that there is little change in ellipticity in the initial $\sim$ 5 ms of instrument downtime. Results for other variants were similar. The single exponential in the unfolding direction can be explained by the low transition state barrier (t_1) between 2U and I_2 relative to the second transition state barrier (t_2). This means that $k_{u1} \gg k_{u2}$, and that k_u therefore approximates to k_{u1} (see Equation 10).

11. Folding measurements were taken between 0.32 and 1.39 M post-mix GuHCl concentrations (see Fig. 4, A and B), a range in which the unfolding rate was not expected to contribute significantly. For the unfolding reactions (Fig. 2B), a 220 μ M solution of folded peptide in 10 mM potassium phosphate, 100 mM potassium fluoride pH 7.0 was mixed against 10 volumes of an appropriate concentration of GuHCl at 293 K and the post-mix GuHCl concentration calculated according to the following: (pre-mix [GuHCl] $\times$ 10)/11. Unfolding measurements were taken between 2.77 and 4.28 M GuHCl (see Fig. 4C) where folding was not predicted to contribute significantly. Unfolding is unimolecular and was found to be independent of protein con-

centration (Fig. 3B). All concentrations of GuHCl dilutions were determined by refractometry. The resulting data points are the result of at least three kinetic transient averages.

Equilibrium Data Analysis—Equilibrium curves were fitted to the two state model in Equation 1,



where N_2 is the native peptide dimer, U is the unfolded monomer, and $K = [U]^2/[N_2]$.

The upper and lower baselines of the unfolding profiles were defined according to Equations 2 and 3,

$$ub = ui + us \cdot [\text{GuHCl}] \quad (\text{Eq. 2})$$

$$lb = li + ls \cdot [\text{GuHCl}] \quad (\text{Eq. 3})$$

where ub and lb are the upper and lower baselines, ui and li are the upper and lower intensities (the respective ellipticities at 0 M denaturant), and us and ls are the upper and lower slopes relating to the unfolded and folded states, respectively. The fraction unfolded can be calculated according to Equation 4,

$$F_U = \frac{\theta_x - lb}{ub - lb} \quad (\text{Eq. 4})$$

to yield the fraction of protein which is unfolded at any given concentration of GuHCl, where θ_x , lb , and ub are the CD signals at point X, for folded protein, and unfolded protein, respectively. Once F_U has been determined for any concentration of denaturant, it may be converted to K_D according to Equation 5,

$$K_D = \left(\frac{2F_U^2 P_t}{1 - F_U} \right) \quad (\text{Eq. 5})$$

where P_t is the total protein concentration (in molar units). For the purposes of the global fitting procedure F_U can be described purely in terms of K_D and P_t . The K_D can then be converted to ΔG for each GuHCl data point according to Equation 6.

$$F_U = \frac{-K_D + \sqrt{K_D^2 + 8K_D P_t}}{4P_t} \quad (\text{Eq. 6})$$

This quadratic function is formed by combining the three equations $K_D = [U]^2/[N_2]$, $P_t = 2[N_2] + [U]$, and $[U] = F_U P_t$ to describe F_U in terms of K_D and P_t only. Values for F_U are calculated using Equations 2–4 (see Fig. 5A), and Equation 6 is solved to derive a value for K_D . To obtain the most accurate value for the free energy of unfolding in water ($\Delta G_{F \rightarrow U(W)}$); however, values for F_U were taken from the transition zone of the denaturation profiles, converted to K_D according to Equation 5, and a linear fit was carried out (Fig. 5B). This is because the signal to noise ratio is at its lowest where the change in intensity is at its greatest, and is achieved by plotting the derived $\ln(K_D)$ as a function of [GuHCl]. A linear fit is used to extrapolate to the free energy of unfolding in water, $\Delta G_{F \rightarrow U(W)}$, in accordance with the linear extrapolation method (32–34) in Equation 7,

$$\Delta G_{U \rightarrow F(W)} = \Delta G_{U \rightarrow F} + m_{eq} \cdot D \quad (\text{Eq. 7})$$

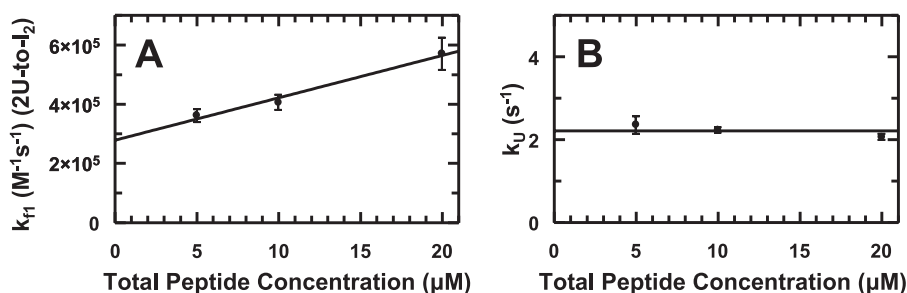


FIGURE 3. The concentration dependence of A, the initial folding amplitude (k_{f1}) and B, the unfolding rate (k_u) for cJun-FosW, performed at 5, 10, and 20 μM P_{H} , respectively, demonstrates that while the initial folding amplitude is concentration-dependent and consistent with a second order reaction, the unfolding amplitude is consistent with a first-order reaction and is independent of concentration.

where $\Delta G_{F \rightarrow U}$ represents the free energy of unfolding at any given concentration of denaturant, and the slope m_{eq} is an approximate measure of the water accessible protein surface exposed per unit concentration of denaturant (with units $\text{cal mol}^{-1} \text{M}^{-1}$).

Thermal Data Analysis—Thermal stability was similarly assessed according to Equations 1–4 and Equation 6. This can then be applied to give a global non-linear fit (shown in Fig. 5C) to derive thermal melting (T_m) values; the temperature at which the protein is 50% unfolded. The T_m values (given in the Abstract) can be derived using a global fit to Equations 2, 3, 4, and 6 and Equation 8,

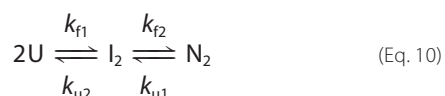
$$\Delta G = \Delta H(T_A/T_m) \times (\Delta H + R \times T_m \times \ln(P_t)) + \Delta C_p \times (T_A - T_m - T_A \times \ln(T_A/T_m)) \quad (\text{Eq. 8})$$

where ΔH is the enthalpy change, T_A is the reference temperature, R is the ideal gas constant, and ΔC_p is the change in heat capacity. Once again to obtain the most accurate estimate of $\Delta G_{F \rightarrow U(w)}$, F_U data corresponding to the transition only were taken, converted to $\ln(K_D)$ according to Equation 5 and plotted against temperature (Fig. 5D) according to Equation 9,

$$\Delta G_{U \rightarrow F(w)} = m \cdot T + C \quad (\text{Eq. 9})$$

where m is the gradient, T is the temperature (in Kelvin) and C is the intercept. Using this fit, a free energy value for the coiled coils at the reference temperature of 293 K could be determined. Measurements for all dimers were started at -8°C , and at this temperature the peptide solutions remained aqueous, even when left overnight. The wild-type protein is unstable and consequently the lower slope associated with the pretransition baseline has been restrained using the average of the other transitions. The restraint in this lower baseline could be varied within the range of known pretransition gradients with variation of only a few degrees centigrade in the calculated T_m .

Kinetic Data Analysis—Kinetic data were fitted to the three state model in Equation 10.



In this model, I_2 represents a dimeric intermediate. In the folding direction, two phases are observed, which is consistent with an intermediate state. Evidence for this intermediate is supported by the fact that the first folding constant (k_{f1}) is bimolecular, being dependent upon the concentration of denatured

peptide (Fig. 3A), which informs that the intermediate state is dimeric. In contrast the unfolding rate is not affected by differences in protein concentration (Fig. 3B), indicating a first-order process. The second folding rate (k_{f2}) is difficult to determine because of its small size relative to k_{f1} . It is clear that the rate constants for these two folding events differ by over five orders of magnitude and have consequently been fitted as uncoupled events according to the following time-

dependent decrease in ellipticity (increase in helicity) in Equations 11 and 12,

$$\theta(t) = \theta_0 + \theta_1 \cdot \left(\frac{1}{1 + (k_{\text{app}} \cdot t)} \right) + (\theta_2 \cdot \exp(-k_{f2} \cdot t)) \quad (\text{Eq. 11})$$

$$k_{\text{app}} = k_{f1} \cdot P_t \quad (\text{Eq. 12})$$

where θ_0 is the final ellipticity, θ_1 is the change in ellipticity associated with the first folding transition, θ_2 is the change in ellipticity associated with the second folding transition ($2U = I_2$), k_{app} is the apparent rate constant for the first folding transition at a given peptide concentration, k_{f2} is the rate constant associated with the second folding transition, and t is time.

In the unfolding direction, only one exponential model is required to fit the kinetic transients, such that the barrier between U and I_2 is Equation 13.

$$\theta(t) = \theta_0 + \theta_1 \cdot (1 - \exp(-k_u \cdot t)) \quad (\text{Eq. 13})$$

In this model (see Equation 10), k_{u1} is slow relative to k_{u2} because of the small size of the transition state barrier that is associated with the first transition, and consequently $k_u \approx k_{u1}$. This unimolecular reaction is not influenced by the concentration of dimer prior to unfolding and is therefore independent of protein concentration (Fig. 3B). This model is supported by equilibrium data collected at 20 μM where no intermediate is detectable; taken together this indicates that the folding barrier between the unfolded state and intermediate is easily surmounted in both directions. Finally, folding data can be fitted as a function of denaturant concentration to yield the kinetic constant of folding and unfolding in 0 M denaturant (w) according to Equations 14–16,

$$\ln(k_{f1(w)}) = \ln(k_{f1}) + (m_u - m_{t1}) \cdot D \quad (\text{Eq. 14})$$

$$\ln(k_{f2(w)}) = \ln(k_{f2}) + (m_i - m_{t2}) \cdot D \quad (\text{Eq. 15})$$

$$\ln(k_{u(w)}) = \ln(k_u) + (m_f - m_{t2}) \cdot D \quad (\text{Eq. 16})$$

where $\ln(k_{f1})$ and $\ln(k_{f2})$ are folding constants associated with first and second transitions accordingly, at any given denaturant concentration, and $\ln(k_u)$ is the unfolding rate at any given final denaturant concentration. Values for m_u , m_{t1} , m_i , m_{t2} , and m_f are m -values associated with each of the identifiable states of the folding pathway and relate to the amount of solvent exposed surface area in each of these states. A fuller explanation is given

in Fig. 6. These equations are used to extrapolate the rate constant and/or free energy of the relevant transition to zero denaturant concentration.

Equilibrium Unfolding—Circular dichroism spectra demonstrated all peptides to be α -helical, with characteristic minima at 208 and 222 nm. GuHCl denaturation profiles were performed at 293 K for all dimeric peptides. All demonstrate a sigmoidal lowering of the 222 nm signal indicative of dimer unfolding. The transition was completely reversible, and the helical signal could be regained upon dilution of the denaturant (data not shown). Values corresponding to the fraction of unfolded protein (F_U) were obtained as described above. Those values around the transition point were converted to ΔG values and extrapolated to the y -axis (see Fig. 5B) to give the most accurate estimation of the free energy of unfolding in water ($\Delta G_{(W)}$). The gradient of this plot corresponds to the m -value (m_{eq}) associated with the overall unfolding transition, and reports upon the exposure of hydrophobic groups to the denaturant in the unfolded state, relative to the folded.

Kinetic Studies—The kinetics of folding were unable to be fitted to a two-state bimolecular model that has been widely reported for GCN4-p1 (12, 14, 17, 35). Fitting to this model did not achieve satisfactory residuals (Fig. 2A). Rather, a biphasic fit that assumed a dimeric intermediate was necessary (Equation 13). Evidence for a dimeric intermediate comes from the fact that the first transition was found to be concentration dependent for the peptide range 5–20 μM for cJun-FosW (data not shown), and further, fitting the data to a monomeric intermediate did not achieve satisfactory residuals. Rather a three state model was proposed with a dimeric intermediate (Equation 10). For all dimers, the first of these two transitions was very fast (Table 1; approximately $10^6 \text{ M}^{-1} \text{ s}^{-1}$, in agreement with previously reported coiled coil folding rates (12, 17)), while the second was much slower ($\sim 1 \text{ s}^{-1}$) suggesting that the two events are not coupled. The unfolding rate, in accordance with a unimolecular reaction, was not concentration-dependent, and was fitted to a two state mechanism.

RESULTS

cFos-JunW_{E23K}—Kinetic parameters for the wild-type molecule could not be obtained, even at 283 K and 40 μM total peptide. This is because of its low overall stability (it is around 3 kcal mol^{-1} less stable than the other variants). All remaining peptides were shown to form a relatively unstable dimeric intermediate with a bimolecular rate constant of between 1.5 and $1.8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (see Table 1 and Fig. 4A). The exception was cFos-JunW_{E23K}, which displayed an accelerated k_{fl} rate constant of $3.8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. Faster folding for this mutant is at first glance somewhat perplexing. The mutation of Glu to Lys at position $g'3$ (see Fig. 1) is predicted to be of slightly lower helical propensity (1.59 *versus* 1.23) according to an experimental study on coiled coil systems that accounts for both solvent-exposed and buried residues, as well as residues located at the center of the helix and at the termini (36). Pairing with Leu at position $e4$ of c-Fos (for notation see Fig. 1) is difficult to rationalize, although it is likely that the additional hydrophobic bulk of Lys adds better core shielding to the dimer and may interact better with Leu on the opposing helix. Additionally, Lys at $g'3$ is

likely to interact much more favorably than Glu with Glu residues at positions $c'3$ and $g'2$ in the same helix, generating additional intrahelical stability. As, at least partial helicity must precede dimerization if monomers are to clash productively (29), it seems likely that the added intramolecular stability of JunW_{E23K}, as well as the additional hydrophobic bulk able to exclude core solvation in the cFos-JunW_{E23K} dimer, is a major driving force in speeding the helix to a conformation capable of dimerization. Indeed, helical content prediction at the residue level using the AGADIR algorithm (37–39) demonstrates that the E23K mutation propagates helical stability across the entire molecule (see Fig. 7).

The unfolding rate for cFos-JunW_{E23K} is very close to that of cFos-JunW suggesting that this mutation plays no role in late, post major transition state, events. A second indication of increased structure in the first transition state of cFos-JunW_{E23K} comes from a lowering of the m -value for the rate constant associated with this transition; a value of -0.5 for the other three molecules lowers to $-0.7 \text{ cal mol}^{-1} \text{ M}^{-1}$ for cFos-JunW_{E23K}, indicating that the molecule has a greater extent of exposed surface area (relative to the folded state) buried at the first transition state ($\sim 36\%$) relative to the other three molecules ($\sim 27\%$). Thus the molecule is more native-like at this stage.

cFos-JunW_{Q21R}—As is shown in Table 1, this mutant displayed a very similar k_{fl} folding rate ($1.6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) to both cFos-JunW (1.5×10^6) and cFos-JunW_{ph1} (1.7×10^6). Collectively this implies that the extra stability afforded to the first transition state for cFos-JunW_{E23K} is compensated by the additional Q21R mutation in cFos-JunW_{ph1}. The mutation does not cause any significant change in the helical propensity (1.27 *versus* 1.21 (36)), but rather appears to generate a better intermolecular partner for $g2$ Glu of c-Fos, by giving a favorable charge-charge interaction (see Fig. 1). It is interesting that the increase in k_{fl} observed for cFos-JunW_{E23K} is not also realized in the cFos-JunW_{ph1} double mutant, especially since the Q21R mutation like the E23K mutation also adds considerable hydrophobic bulk to the dimeric interface. This implies that the Q21R mutation neutralizes the E23K mutation at this step and prevents this faster folding rate from being realized. This may be explained by intrahelical repulsions introduced at the $e'3$ position with neighboring $e'2$ and $e'4$ residues Lys and Arg. the side-chains of $e'2$ – $e'3$ and $e'3$ – $e'4$ are $\sim 10 \text{ \AA}$ apart in space according to the crystal structure of the wild-type molecule (40). In agreement with Coulomb's law this $10 \text{ \AA}$ distance would be predicted to contribute $\sim 0.4 \text{ kcal mol}^{-1}$ of unfavorable potential energy to the molecule. This is also supported by helical content prediction at the residue level by AGADIR (37–39) (see Fig. 7) which demonstrates JunW_{Q21R} to have decreased helical content, at the C-terminal region, relative to JunW.

The $E_{g2}Q_{e'3} \rightarrow E_{g2}R_{e'3}$ interhelical interaction that arises from the Q21R mutation is predicted to have a favorable $\Delta\Delta G$ of $-0.6 \text{ kcal mol}^{-1}$ (41). Because this mutation has little or no effect on the initial fast transient, it seems likely that the formation of the Coulombic interaction occurs late in the folding process, thus accounting for the slower unfolding rate when it is strengthened. The fact that the mutation has no effect upon folding can be explained by the intramolecular repulsion pro-

vided by surrounding $e'2$ Lys and $e'4$ Arg. Again, this may explain why the faster folding of the E23K mutation is only partially realized in the double mutant JunW_{Ph1}. Whereas Gln would be predicted to interact well with these two residues, Arg almost certainly results in steric repulsion with both. Together with the E23K mutant, which has the opposite effect of strengthened intramolecular contacts with neighboring residues (*i.e.* with $g'2$ Glu as well as $c'3$ Glu), these data together imply that intramolecular (*i.e.* helical) and possibly hydrophobic interactions are formed early on in the folding process, whereas intermolecular Coulombic interactions are consolidated later on in the folding process, after the major transition state has been traversed. This is logical when one considers that one helix must have a degree of order before it is able to specifically recognize and bind to the second. Again, an initial hydrophobic collapse would be consistent with this model. Later intermolecular charge-charge interactions can only be achieved when a large degree of organized folded structure has already been realized. Once again a study of the m -values consolidates this interpretation; whereas the other three dimeric molecules display m -values of 1.04–1.08 cal mol⁻¹ M⁻¹ associated with the F-to-mt₂ transition, cFos-JunW_{Q21R} displays an m -value of 1.4 cal mol⁻¹ M⁻¹, indicating that relative to the fully folded state, the amount of exposed surface area is greater at m_{t2} for this molecule (~78% exposed) than for the others (56%). This correlates with the extra $E_{g2} \leftrightarrow R_{e'3}$ interaction that has yet to be satisfied at this point in the pathway. It is likely that the intramolecular steric repulsions also introduced with this mutation lead to a lack of overall stability for this mutant, and is also why the combination of the Q21R and E23K mutations only gives rise to a small increase in k_{f1} for cFos-JunW_{Ph1}. Similarly the thermodynamic stability afforded by the Q21R mutation is only marginally realized in the double mutant when analyzing k_{u1} . One explanation is that the additional stability revealed by thermal melting data is accounted for by the changes in k_{f2} or k_{u2} , which were unable to be determined. These thermal melting data (see Table 2 and Fig. 5) performed at 20 μ M and 150 μ M demonstrate the double mutant dimer to be around 1.6 kcal mol⁻¹ more stable than cFos-JunW_{E23K} and 0.8 kcal mol⁻¹ more stable than cFos-JunW_{Q21R}.

cFos-JunW_{Ph1}—The double mutant cFos-JunW_{Ph1} did not exhibit the fast initial phase displayed by cFos-JunW_{E23K} because the improved $g'2 \leftrightarrow g'3$ intramolecular contacts in JunW_{E23K} ($EE \leftrightarrow EK$) that contribute to an increased folding rate are compensated for by weakened $e'3 \leftrightarrow e'4$ ($QR \leftrightarrow RR$) and $e'2 \leftrightarrow g'3$ ($KQ \leftrightarrow KR$) intramolecular contacts in JunW_{Q21R}, which would be predicted to lower intramolecular stability.

The double mutant JunW_{Ph1} does however partially retain the slower unfolding rate that is exhibited by cFos-JunW_{Q21R}. In the first instance, the additional free energy (−0.6 kcal mol⁻¹) contributed by the $EQ \rightarrow ER$ charge interaction late in the JunW_{Q21R} folding pathway is only partially compensated for by the E23K mutation ($LE \rightarrow LK$ represents a gain of two methylene groups and would be predicted to increase core shielding. The major effect of the E23K mutation, however, is increased intra-helical stability. Consequently, a fast initial collapse is observed as helices form and dimerize. This initial fast formation of helicity is compromised in Q21R, which lowers

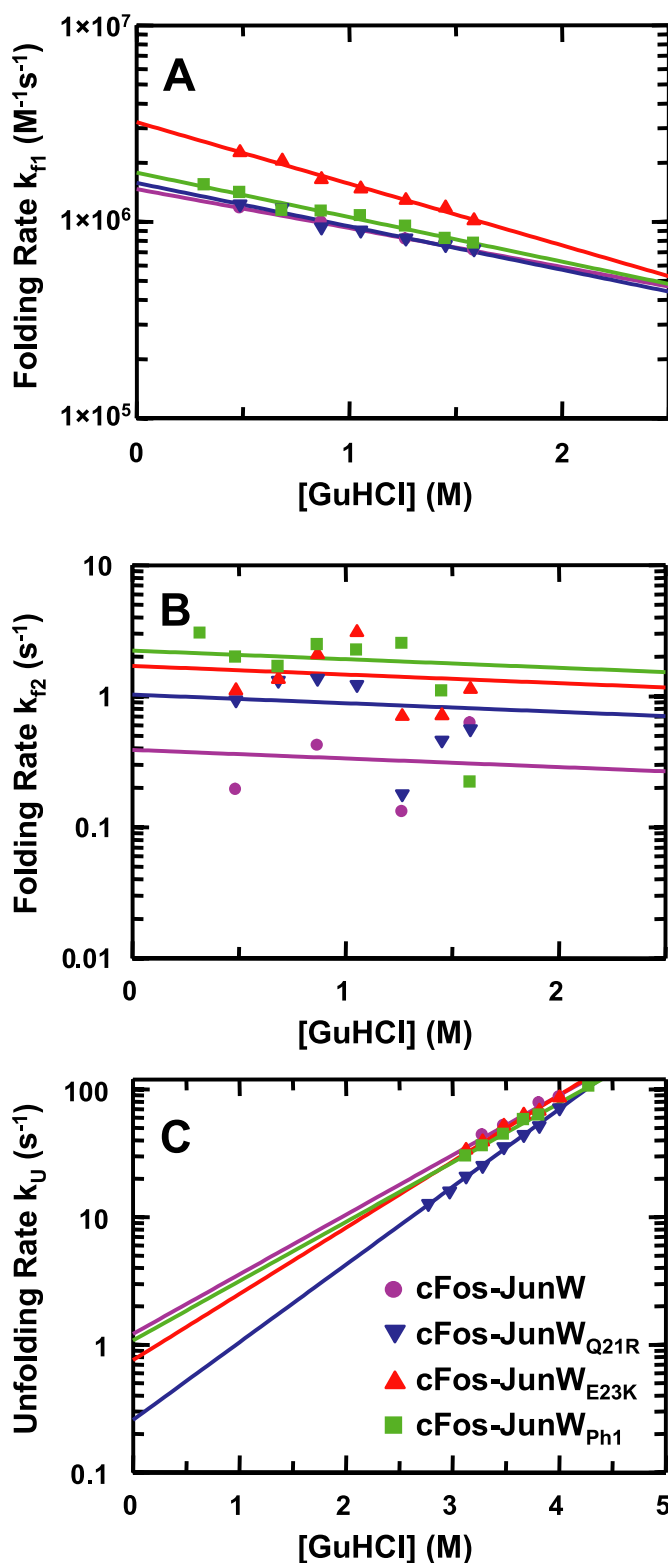


FIGURE 4. GuHCl dependence of the rate constants for refolding (A, k_{f1} ; B, k_{f2}) and unfolding (C, k_{u1}). Values for k_{u2} cannot be obtained with any degree of certainty because of the large error in k_{f2} . This error results from the large differences in rate between k_{f1} and k_{f2} , so that while the initial fast rate can be accurately determined, the second cannot (see B). Data for k_{f2} have not been used in the interpretation of results but are included here for completeness; in this case, m -values have been restrained to fall exactly halfway between m -values for $t1$ and $t2$, this range represents around 15% of the overall m -value change. Lines represent global fits to the data, with each data point being the average of at least three kinetic transients. In the case of A, k_{app} has been corrected for peptide concentration according to Equation 12.

intrahelical interactions with surrounding residues (*i.e.* with e'2 K and e'4 R). Consequently, the combining of these two mutations results in little overall effect on the first folding transitions, but retains partially slower unfolding. It is interesting as to why the partial lowering of the unfolding rate in the double mutant is not reflected in the *m*-value.

While m_w , m_{t1} , m_{t2} , and m_f (approximated by combining equilibrium data with kinetic data) are known, m_{12} is not. This is because of the fact that the relationship between [GuHCl] and k_{t2} is unreliable (see Fig. 4B), and that data for k_{u2} cannot be calculated. However, subtracting *m*-values associated with k_{f1} and k_{u1} from our value for m_{eq} (derived using GuHCl denaturation data; see also Fig. 6) shows that the change in *m*-value associated with the m_{t1} -to- m_{t2} transition is around -0.3 for all mutants. Collectively, it can be calculated from the *m*-values associated with k_{u1} (see Table 1 and Fig. 6) that only around 44% of the native structure is achieved by the time the second transition state (t_2) is reached, more surprising is that only around 27% of the hydrophobic burial is achieved at the time of the first transition state (t_1). This implies that the structure of both transition states must be remarkably similar and that the majority of hydrophobic exclusion does not occur until after the second transition state is traversed. This is interesting since the change in ellipticity demonstrates that the majority of helicity is attained within the first phase (*i.e.* by m_{t1}). It would therefore appear that while the individual monomers are almost fully helical at this point, they are far from native-like as a dimeric species (42). The remainder of the folding pathway must therefore be spent optimizing quaternary structure *e.g.* intermolecular contacts that are seen to form after the second transition state in the cFos-JunW_{Q21R} dimer as well as optimizing hydrophobic packing and solvent exclusion from the hydrophobic helix-helix interface. This is in agreement with a hierarchical model of folding where local interactions dominate early events and the native structure forms from smaller structures (43, 44). Indeed the larger *m*-value for the m_f -to- m_{t2} transition for this mutant suggests m_{t1} and m_{t2} are almost identical in terms of hydrophobic exposure, and that formation of this additional intermolecular electrostatic interaction makes a major contribution.

DISCUSSION

The Fos-Jun transcription factor AP-1 plays a central role in signaling pathways and is deregulated in tumors (25, 26). Pep-

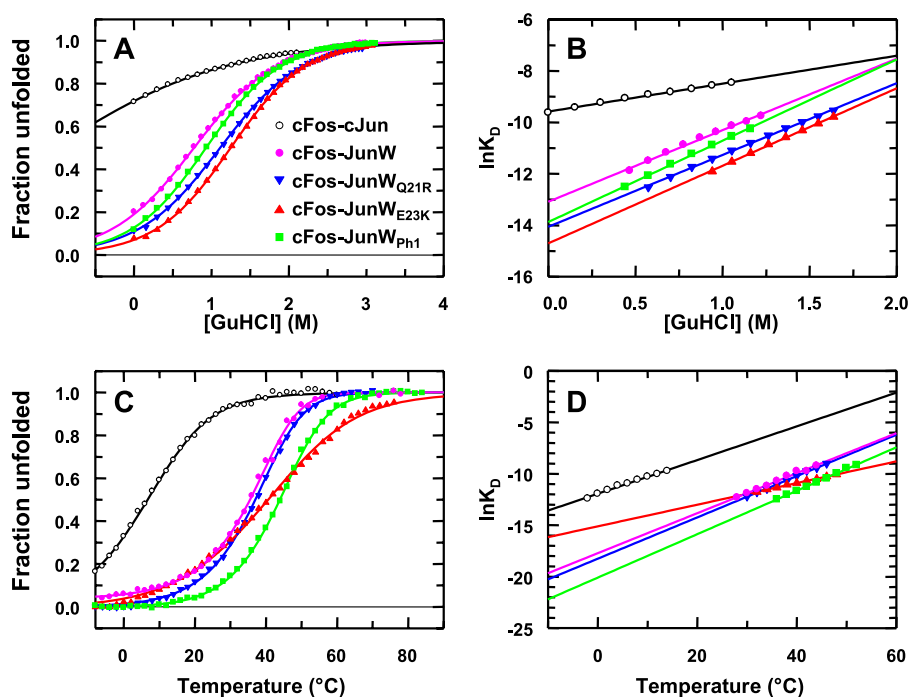


FIGURE 5. **Equilibrium and thermal denaturation profiles.** 20 μ M total peptide. A, equilibrium denaturation profiles carried out at 20 μ M total peptide. B, linear fit to the transition zone region of data in Fig. 4A to determine the K_D at 0 M GuHCl ($\Delta G_F \rightarrow U$ (equilibrium); see Equation 7 and Table 2). C, thermal melting profile carried out at 20 μ M total peptide. D, linear fit to the transition zone of data in Fig. 4C to determine the K_D at 293 K according to Equation 9. Experiments were undertaken in a 1 cm CD cell, and overall ellipticity was monitored at 222 nm. Note that thermal melting data (C) is required to achieve satisfactory lower baselines necessary to give a reliable fit to the data. ΔG values obtained from thermal melting data are normalized to be independent of peptide concentration (see Equation 5), and only data from around the midpoint of the transition are used to give the most reliable K_D estimate. Data for 150 μ M thermal melts are not shown but agree well with thermal melting data obtained at 20 μ M (See Table 2). Because the wild-type molecule is unstable and lacks a pretransition state baseline for thermal denaturation, it was necessary to restrain the fitting procedure using an average of the lower slopes for the other molecules.

tides that target the leucine zipper dimerization domain and modulate transcription factor function are consequently powerful tools for dissecting cellular pathways and in developing anticancer drugs (45, 46). In addition, a firm understanding of rules that give rise to stable and specific peptide-peptide interactions is of great use in designing and engineering proteins (47, 48). We have elucidated the mechanism of coiled coil assembly by dissecting the folding pathway of Fos-Jun based peptides.

The instability of the wild-type molecule cFos-cJun (29, 49) did not permit the kinetic parameters to be extracted, even at low temperatures and high peptide concentrations. This was not surprising, because a full melting profile could not be achieved even at 150 μ M total peptide concentration. Nonetheless comparison of cFos-JunW, cFos-JunW_{Ph1}, and the two intermediate mutants cFos-JunW_{Q21R} and cFos-JunW_{E23K} permitted us to assess the contributions of these residues to identifiable kinetic steps in the folding pathway. JunW_{E23K} resulted in increased intramolecular stability ($K_{g'3} \leftrightarrow E_{g'2}$, $K_{g'3} \leftrightarrow E_{c'3}$; see Fig. 1) and a greater potential for hydrophobic burial in the heterodimer; this is because the side-chain of Lys has two additional methylene groups relative to Glu and therefore generates improved shielding of the closely packed *a/d* residues from the solvent. Both factors led to an increased rate of formation of the initial dimeric intermediate.

In contrast, JunW_{Q21R} led to a decrease in the rate of unfolding from the fully folded heterodimer to the dimeric interme-

diolate. This is accountable to an additional interhelical Coulombic interaction with cFos ($E_{g2} \leftrightarrow R_{e'3}$; see Fig. 1) that is less favored for the wild-type ($E_{g2} \leftrightarrow A_{e'3}$) or cFos-JunW ($E_{g2} \leftrightarrow Q_{e'3}$) dimer (41). Indeed, even Asn-Asn pairs buried in the core of GCN4 have been shown to exert their effect on the molecule late in the folding pathway (35). However, the same mutation also destabilizes the helix by forming intrahelical charge-charge repulsions with neighboring e' residues Lys and Arg, preventing accelerated folding. Thus, when the E23K and Q21R muta-

tions are combined, a double mutant (JunW_{Ph1}) stabilized by faster folding and slower unfolding is only partially observed (see Table 1 and Fig. 4). Collectively this implies that interhelical interactions for protein-protein interactions are formed late in folding, after the rate-limiting transition state. This is supported by studies on GCN4 which suggests interhelical salt-bridges stabilize the molecule but do not accelerate folding (50).

The dimeric complex cFos-JunW_{Ph1} displays a partially faster folding rate relative to cFos-JunW and cFos-JunW_{Q21R}. The combined mutant also displays a partial slowing of the unfolding rate relative to cFos-JunW and cFos-JunW_{E23K}. The result correlates well with thermal unfolding data performed at both 20 μ M and 150 μ M (see Table 2), but correlates less well with equilibrium denaturation data where satisfactory lower baselines were not observed (see Fig. 3A). Equilibrium derived free energy values have therefore been included for completeness but have not been used in the interpretation. The overall picture to emerge from thermal melting data is that of an unstable wild-type molecule (5 kcal mol⁻¹; see Table 2), with cFos-JunW stabilized (8 kcal mol⁻¹). Relative to this stabilized molecule, cFos-JunW_{Q21R} is more stable still (8.5 kcal mol⁻¹), and cFos-JunW_{E23K} is partially destabilized (7.6 kcal mol⁻¹). The double mutant cFos-JunW_{Ph1} is more stable (9.2 kcal mol⁻¹) than either of the two intermediate mutants. When creating coiled coils of increased stability, designing for thermodynamic stability by engineering decelerated unfolding rates would appear to be the most logical approach. This is especially true of Jun-Fos folding, where the initial folding amplitude does not represent the formation of the folded state, and little information can be extracted from the second phase. In contrast, for the unfolding direction there is only one major transition state to traverse. This outcome suggests that carefully selecting mutations that maintain helix propensity and intramolecular interactions, but increase intermolecular electrostatic contributions, can make large increases to the free energy of unfolding and are relatively easy to design.

Another central finding of this study is that different selections yield different peptides.⁴ While both protein-fragment complementation assays (PCA) (51–53) and phage display systems express proteins in *Escherichia coli*, they have fundamental differences; phage display requires proteins to locate and fold in the periplasm, and selection occurs *in vitro* under artificial conditions. By contrast, in PCA, expression and selection occurs in the cytoplasm of *E. coli* in the presence of a vast number of cellular proteins, perhaps these extra constraints for PCA explain why a T_m comparable to the phage display technique was unable to be achieved.

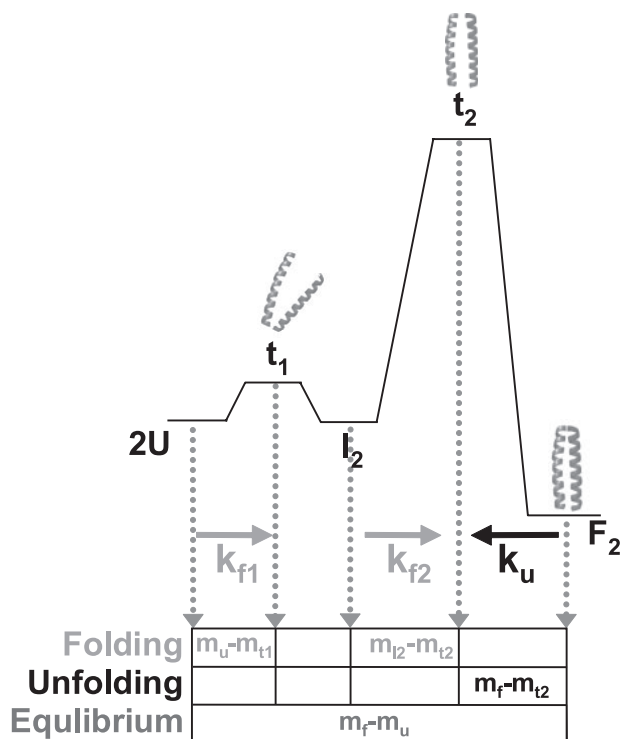
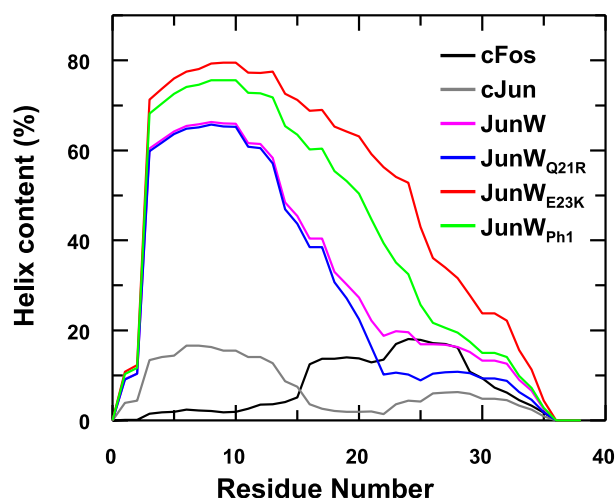


FIGURE 6. Free energy diagram highlighting the identifiable steps in the folding pathway of AP-1. Rate constants k_{f1} and k_{f2} are determined by the height of the small and large transition state barrier from 2U and I_2 , respectively. Likewise the height of the transition state barrier from F_2 in the unfolding direction dictates the value of k_u . This value in turn approximates to k_u because of the small second transition state that is easily traversed. m -values associated with the transitions (according to Equations 14–16) are also shown, as is the overall m -value from equilibrium. Note that because k_{f2} is not accurately determined and k_{u2} cannot be determined at all, this leaves no known value for m_{t2} that can be determined. Rather we are left with values for m_u , m_{t1} , m_{t2} , and an estimate of m_f (from equilibrium data). Shown above are schematics of the molecule; at the denatured state the helices are almost entirely random coil. By t_1 the monomers are almost entirely helical with partial hydrophobic exclusion (27% of native state exclusion) from the solvent via interactions at the hydrophobic interface. At t_2 , this hydrophobic exclusion has increased little (44% of native exclusion). Finally, only after this state do the favorable intermolecular Coulombic interactions form and the molecule arrives at the native state.

TABLE 1
Kinetic folding data

Shown are the kinetic data associated with each of the identifiable transitions. The first column is associated with the 2U-to- I_2 transition, the second with the I_2 -to- F_2 transition, and the third with the F_2 -to-2U transition. The rate constants and m -values associated with these transitions are derived from Equations 12–14, and displayed in Fig. 4.

Mutant	Folding 1 st exp		Folding 2 nd exp		Unfolding	
	$k_{f(w)}$ $s^{-1} M^{-1}$	m_u -to- m_{t1}	$k_{f(w)}$ s^{-1}	m_{t2} -to- m_{t2} (restrained)	k_u s^{-1}	m -value $cal\ mol^{-1}\ M^{-1}$ ($-m_{t2}$)
cFos-JunW	$1.47e6 \pm 1.72e4$	-0.46 ± 0.01	3.39 ± 0.41	-0.63	1.21 ± 0.49	1.08 ± 0.11
cFos-JunW _{Q21R}	$1.56e6 \pm 7.82e4$	-0.51 ± 0.05	0.56 ± 0.26	-0.63	0.26 ± 0.03	1.40 ± 0.04
cFos-JunW _{E23K}	$3.22e6 \pm 1.10e5$	-0.72 ± 0.04	1.68 ± 0.26	-0.63	1.31 ± 0.29	1.04 ± 0.06
cFos-JW _{Ph1}	$1.78e6 \pm 6.04e4$	-0.52 ± 0.04	2.67 ± 0.60	-0.63	1.08 ± 0.12	1.07 ± 0.03



cFos: ASTDTLQAEITDQLEDEKYALQTEIANLLKEKEKLGAP
cJun: ASIARLEEKVKTLKAQNYELASTANMLREQVAQLGAP
JunW: ASAAELEERVKTLKAEIYELQSEANMLREQIAQLGAP
JunW_{Q21R}: ASAAELEERVKTLKAEIYELRSEANMLREQIAQLGAP
JunW_{E23K}: ASAAELEERVKTLKAEIYELQSKANMLREQIAQLGAP
JunW_{Ph1}: ASAAELEERVKTLKAEIYELRSKANMLREQIAQLGAP

FIGURE 7. Helical content prediction at the residue level as calculated by AGADIR (37–39). c-Fos (black line) is of lowest helical propensity throughout the sequence and naturally pairs with c-Jun (gray). JunW (magenta) is of improved helical propensity, with the Q21R mutation (JunW_{Q21R}; blue) causing a lowering in propensity relative to JunW, notably around the mutation at the C terminus of the helix. In contrast, the E23K mutation found in both JunW_{E23K} (red) and JunW_{Ph1} (green) exerts its effect of increased helical propensity across the entire helix. Shown below are the sequences corresponding to the above residue number. For the latter three sequences the Q21R and E23K mutations relative to JunW are bold and underlined.

No sequences have been identified that conform to a speculated “trigger sequence” for folding initiation (18, 54). Rather, overall helical propensity enforces α -helical topology early in the folding pathway to give rise to structures that are able to associate within a realistic time frame. This notion is supported by helical content predictions using the algorithm AGADIR (37–39). AGADIR is based on an empirical analysis of experimental data and estimates energy contributions (such as intrinsic helical propensities, side-chain to side-chain interactions, main-chain to main-chain hydrogen bonds, and capping effects) to account for the stability of isolated α -helices. Specifically, it predicts JunW_{Q21R} to have a lower helical content than JunW in the C-terminal region where the mutation is found (see Fig. 7). In contrast, the E23K mutation resulted in high helicity for both JunW_{E23K} and JunW_{Ph1}. This occurred not only in the locality of the mutation, but over the entire helix, particularly in the N-terminal region, (see Fig. 7). Together with the Q21R mutation, this leads one to speculate that it is the N-terminal region of the Jun molecule that is helical first, and hence is responsible for initiating early folding events. Indeed, inspection of helical content at the residue level for cJun and cFos implies that the former initiates N-terminal folding of the molecule, and the latter C-terminal folding. In combination with charge repulsions in the cFos homodimer, this may support the explanation as to why the cFos-cJun heterodimer forms preferentially over either homodimer. With regard to folding data, the initial folding rate, k_{u1} , correlates well with helical content predictions; JunW_{E23K} has the fastest initial

TABLE 2

Free energy values of unfolding

Equilibrium derived values are performed at 20 μ M total peptide concentration and 293 K. Thermal values have been collected at both 20 μ M and 150 μ M total peptide concentrations using a reference temperature of 293 K. In all instances, a plot of $\ln K_D$ vs. either [GuHCl] or temperature using fraction unfolded (F_U) data from the transition point only was used to give the best estimate of $\ln(K_D)$ at the reference temperature and 0 M GuHCl (Equation 5; data not shown). It should be noted that the lack of a reliable lower baseline for GuHCl denaturation data (See Fig. 5A) meant that only thermal denaturation data (See Fig. 5, C and D) was used in the interpretation.

Mutant	m_{eq}	$\Delta G_{F \rightarrow U}$ (equilibrium)	$\Delta G_{F \rightarrow U}$ (20 μ M thermal)	$\Delta G_{F \rightarrow U}$ (150 μ M thermal)
cJun-cFos	-0.63	5.5	5.0	4.7
cFos-JunW	-1.84	7.7	8.1	7.8
cFos-JunW _{Q21R}	-1.80	8.1	8.3	8.6
cFos-JunW _{E23K}	-1.98	8.4	7.6	—
cFos-JunW _{Ph1}	-1.87	8.0	9.3	9.1

folding rate, followed by JunW_{Ph1}, which is faster than either JunW_{Q21R} or JunW. In contrast to a previous study (17), these data collectively support the argument that helices are native-like in secondary structure prior to their collision (42).

The overall picture of folding to emerge (see Fig. 6) is that of a coiled coil which has gained almost all of its native helicity at the time of the rapidly formed dimeric intermediate. It is the high helical content as well as hydrophobic interactions at the helical interface that permits dimerization of monomers and, hence, arrival at this state. However, for this intermediate to progress to the folded state, more intricate interactions must be formed, particularly interhelical Coulombic interactions. These can become correctly aligned after the second major transition state has been traversed, resulting in a molecule which is both stable and specific in its dimeric contacts. This second major transition state barrier is also likely to represent adjustments in packing of the hydrophobic interface which optimizes van der Waals interactions and concomitantly dehydrates the core by expelling residual water molecules (55, 30).

In summary, engineering increased folding rates by conserved substitution of helix stabilizing mutations can accelerate the path to the folded state. In contrast optimizing intermolecular Coulombic interactions can generate increased thermodynamic stability, thus ensuring that the unfolded state is inaccessible to preformed complexes. These rules can be further tested to generate specific, stable peptides and peptide-based compounds for use in therapeutics and nanobiotechnology.

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