An engineered leucine zipper *a* position mutant with an unusual three-state unfolding pathway

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HAI ZHU,¹ SCOTT A. CELINSKI,² J. MARTIN SCHOLTZ,^{1,2} AND JAMES C. HU¹

¹Department of Biochemistry and Biophysics, Center for Advanced Biomolecular Research, Texas A&M University, College Station, Texas 77843, USA

²Department of Medical Biochemistry and Genetics, Center for Advanced Biomolecular Research, Texas A&M University, College Station, Texas 77843, USA

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Abstract

The leucine zipper is a dimeric coiled-coil structural motif consisting of four to six heptad repeats, designated $(abcdefg)_n$. In the GCN4 leucine zipper, *a* position 16 in the third heptad is occupied by an Asn residue whereas the other *a* positions are Val residues. Recently, we have constructed variants of the GCN4 leucine zipper in which the *a* position Val residues were replaced by Ile. The folding and unfolding of the wild-type GCN4 leucine zipper and the Val to Ile variant both adhere to a simple two-state mechanism. In this study, another variant of the GCN4 leucine zipper was constructed by moving the single Asn residue from *a* position 16 to *a* position 9. This switch causes the thermal unfolding of the GCN4 leucine zipper to become three state. The unfolding pathway of this variant was determined by thermal denaturation, limited proteinase K digestion, and sedimentation equilibrium analysis. Our data are consistent with a model in which the variant first unfolds from its N terminus and changes the oligomerization specificity from a native dimer to a partially unfolded intermediate containing a mixture of dimers and trimers and then completely unfolds to unstructured monomers.

Keywords: Leucine zippers; protein folding; folding intermediate; three-state unfolding

Supplemental material: See www.proteinscience.org.

A major challenge in protein folding is to change a protein folding pathway by simply switching one or two amino acids (Creighton 1974; Privalov 1979; Baldwin 1989). Generally, small proteins are favored in protein folding studies because of their well-defined characteristics and simplicity. Thus, finding a protein, especially a simple small protein, whose folding pathway can be altered would be very useful for addressing some fundamental issues in protein folding.

Leucine zippers are short peptides that form dimeric coiled coils. The leucine zipper is characterized by a heptad repeat of leucine residues (Landschulz et al. 1988), in which each position in the heptad is identified following a conventional nomenclature for coiled coils, $(abcdefg)_n$ (McLachlan and Stewart 1975). In leucine zippers, the *d* positions are almost always occupied by leucine, the *a* positions are usually comprised of β -branched residues (Hurst 1994), and the residues at the *a* and *d* positions from the two monomers pack to create a hydrophobic interface. In addition to the hydrophobic residues in the dimerization interface, there is often a polar Asn residue at a single *a* position. Although the presence of the polar Asn residue decreases the stability of leucine zippers, two Asn residues from different monomers form an interhelical hydrogen bond to facilitate the formation of specific dimers (O'Shea et al. 1991; Alber 1992; Harbury et al. 1993; Potekhin et al. 1994; Zeng and Hu 1997). There are a few leucine zippers with these

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Reprint requests to: James C. Hu, Department of Biochemistry and Biophysics, Center for Advanced Biomolecular Research, Texas A&M University, College Station, TX 77843-2128, USA; e-mail: jimhu@tamu.edu; fax: 979-845-9274.

Present address: Bioinformatics Department, Pioneer Hi-bred, 7250 NW 62nd Ave, P.O. Box 552, Johnston IA 50131-0552.

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buried Asn residues close to either the N terminus or the C terminus, although usually they are found in one of the central heptads (Hurst 1994). The reason for this positional preference is unknown, but it may be important for the interaction specificity between different leucine zipper proteins (McKnight 1991).

The folding of most dimeric leucine zippers characterized thus far show that folding and dimerization are tightly coupled and very cooperative (Qian 1994; Thompson et al. 1993). The GCN4 leucine zipper, a well-characterized short peptide (33-amino acid residues), shows the typical two-state unfolding in equilibrium experiments. Here, we report that a GCN4 variant constructed by moving an Asn residue from a position 16 to a position 9 shows multiple-state unfolding behavior with a change in the oligomerization state during unfolding.

Results

Sequences of a position mutants of the GCN4 leucine zipper

Two *a* position mutants of the GCN4 leucine zipper, MNIII and MINII, were used to explore the position effects of the buried polar Asn residue on the folding of leucine zippers. The design of these two mutants was described previously (Zhu et al. 2000), and their sequences are shown in Figure 1. The sequences of these two mutants differ from the wild-type GCN4 leucine zipper at *a* positions 9, 16, 23, and 30. Like the wild-type GCN4 leucine zipper, the MINII variant, which is used here as a control, has an Asn at the central *a* position (position 16), whereas in MNIII, the Asn residue was moved to the second *a* position (position 9). The first *a* position is always Met, and the other three *a* positions in both the peptides are occupied by Ile residues. The MINII variant behaves like the wild-type GCN4 leucine zipper,

Fig. 1. Sequences of the GCN4 leucine zipper variants investigated here. Lowercase letters above the sequence indicate the position in the heptad. The *a* positions are highlighted in black, and the *d* position altered is highlighted in gray. The numbering scheme used is intended to maintain consistency with previous GCN4 peptides (O'Shea et al. 1989). The nomenclature of the mutants is based on the sequence of the peptides at the *a* (and in some cases *d*) positions.

showing simple two-state unfolding in thermal denaturation experiments (Zhu et al. 2000). Both variants form stable, dimeric coiled coils at low temperature as indicated by circular dichroism and sedimentation equilibrium experiments (see below).

Urea denaturation of the *a* position variants

The stability of each of the peptides was determined by measuring the ellipticity at 222 nm as a function of urea concentration from 0 to 10 M at 25°C (Fig. 2). The total peptide concentration in each experiment was 20 μ M in terms of monomers. The unfolding of the MINII mutant is highly cooperative and shows two-state behavior. The unfolding of the MNIII mutant is much less cooperative than MINII, although it also shows a single unfolding transition. A two-state model (equations 2–4), which assumes that the unfolding and dissociation are coupled (Thompson et al. 1993; Gloss and Matthews 1997), was used to calculate the parameters shown in Table 1. MNIII has a much smaller *m* value than the MINII, whereas it has a higher *C_m* value. Because there is a correlation between *m* values and the change in surface area upon unfolding (Myers et al. 1995), MNIII and MINII should have the very similar *m* values. The observed differences in *m* value could reflect the presence of residual structure in the denatured ensemble (Shortle 1995). Alternatively, the decrease in the *m* value for MNIII could indicate that its unfolding deviates from a two-state process (Pace 1975; Spudich and Marqusee 2000); we favor this latter explanation based on the results presented below.

Fig. 2. Urea denaturation experiments for the *a* position variants. The unfolding transitions were monitored by circular dichroism at 222 nm in 10 mM phosphate buffer. The peptide concentration was 20 μ M total in terms of monomers. The mutants shown are MINII (circles) and MNIII (squares). The curves on the plot were generated from the parameters listed in Table 1.

Table 1. Analysis of urea denaturation curves of different variants^a

Variant	C _m (M urea)	$\Delta G_{\text{H}_2\text{O}}^0$ (25°C) (kcal mol ⁻¹)	<i>m</i> value (kcal mol ⁻¹ M ⁻¹)
MNIII	6.6 ± 0.2	9.7 ± 0.1	0.56 ± 0.05
MINII	6.0 ± 0.2	11.4 ± 0.1	0.84 ± 0.05

^a $\Delta G_{\text{H}_2\text{O}}^0$ and *m* values were obtained by fitting equations 2–4 to the urea denaturation curves. C_m was determined from the first derivative of the curves.

Thermal denaturation of the MMNIII variant

Thermal denaturations were performed on the MNIII and MINII peptides at a concentration of 20 μM in terms of monomers (Fig. 3). The thermal unfolding of MINII follows a single cooperative unfolding transition, similar to that observed for the wild-type GCN4 leucine zipper. In contrast, the thermal unfolding of the MNIII peptide is clearly not two state; a partially folded intermediate is populated between 50°C and 65°C.

To further characterize the unfolding of MNIII, thermal denaturation was performed at different peptide concentrations. The unfolding of the peptide was monitored by circular dichroism as a function of temperature from 0°C to 100°C (Fig. 4). As illustrated in Figure 4A, the two unfolding transitions for MNIII are concentration dependent, but in opposing directions. For the second transition, the melt-

ing point, T_{m2}, increases as the peptide concentration increases (Fig. 4A), consistent with a dissociation-coupled unfolding, in which a higher-order oligomer in the intermediate is unfolded to a monomeric unfolded state. The concentration dependence of the first transition is especially evident at higher peptide concentrations. Because we had to use very high peptide concentrations, ellipticity at 234.5 nm (on the edge of the strong band with a minimum at 222 nm) was used to monitor the first transition of the unfolding of MNIII (Fig. 4B). As the peptide concentration increases, the midpoint of this first transition (T_{m1}) decreases (Fig. 4B). This suggests that the first transition is an association-coupled partial unfolding, with a lower-order oligomer partially unfolding and associating to a higher-order species. Because we know that the MNIII mutant is a dimer at low temperature (4°C) (Table 2) and the completely unfolded leucine zipper is a monomer, we conclude that the thermal denaturation of the MNIII mutant occurs in two stages with a partially unfolded, oligomeric intermediate of order greater than two. Because the thermal denaturation of MINII is two state from folded dimer to unfolded monomers, the simple switch of the Asn residue from *a* position 16 to 9 has altered the folding pathway.

The unfolding pathway of the MNIII variant

To determine what part of the MNIII peptide is unfolded in the intermediate, limited proteinase K digestion was per-

Fig. 3. Thermal denaturation experiments of the *a* position variants. The unfolding transitions were monitored by circular dichroism at 222 nm in 10 mM phosphate buffer. The peptide concentration was 20 μM total in terms of monomers. The mutants shown are MINII (circles) and MNIII (squares).

Fig. 4. Thermal denaturation experiments of MNIII at different peptide concentrations. (A) The unfolding transitions were monitored at 222 nm, and the peptide concentrations are 2.5 μM (circles), 5.0 μM (squares), 10 μM (diamonds), 20 μM (triangles), and 40 μM ($\times$). (Inset) The plot of the second transition midpoint T_{m2} against the total peptide concentration. The curve in the inset is a fit to a logarithmic relationship. (B) The unfolding transitions were monitored at 234.5 nm, and the peptide concentrations are 0.3 mM (circles), 0.6 mM (squares), and 1.2 mM (diamonds). (Inset) The plot of the first transition midpoint T_{m1} plotted against the total peptide concentration. The T_{mS} were determined from the derivatives of the thermal unfolding curves. The curve in the inset is a fit to a logarithmic relationship.

formed on the MNIII peptide under conditions in which the intermediate is populated. As a control, the same limited proteolysis was performed on the MINII peptide under identical conditions. The reactions were monitored by MALDI-TOF mass spectrometry. For MNIII, a major fragment with a molecular mass of 3378 daltons was obtained (see Materials and Methods). This fragment remains stable even after

24 hours in the presence of the proteinase. In contrast, the MINII peptide is resistant to the action of proteinase K, and it remains intact after 24 hours. Because proteinase K is a nonspecific protease (Blacklow et al. 1995), the difference in the limited digestion properties between MNIII and MINII is most likely caused by local structural differences between those two peptides.

Table 2. The apparent molecular weights of different peptides at different temperatures obtained by fitting sedimentation equilibrium data to a single species model^a

Peptide	Temperature (°C)	Monomer Molecular Weight (Dalton)	Apparent Molecular Weight (Dalton)
MNIII	4	4,203	8,400
	37	—	10,200
NIII ₆₋₃₃	4	3,336	7,700
MG _{d5} NIII	4	4,147	10,000
MA _{d5} NIII	4	4,161	9,700

^a The peptides are named as shown in Figure 1. The apparent molecular weight was determined from the analysis of analytical ultracentrifugation studies as described in the text. Additional data and analyses are available as Supplemental Material (see www.proteinscience.org).

The major fragment from the limited digestion of the MNIII peptide was purified, and its N terminus was determined by Edman degradation to be Glu-Asp-Lys-Asn-Glu. Based on the molecular weight of the fragment, the N-terminal sequence, and the known sequence of the MNIII peptide, the stable fragment was identified as NIII₆₋₃₃ (Fig. 1), which is derived from the MNIII peptide by removing the first seven N-terminal residues.

If the first N-terminal heptad of the peptide unfolds independently from the rest of the peptide, a mutation at *d* position 5 that destroys the hydrophobic packing of the first heptad should only affect one of the two observed unfolding

transitions. To test this hypothesis, we constructed two additional variants, MG_{d5}NIII and MA_{d5}NIII (Fig. 1). These variants replace the Leu in the first heptad (*d* position 5) with a Gly or an Ala residue, respectively.

The NIII₆₋₃₃, MG_{d5}NIII, and MA_{d5}NIII peptides all form α -helical oligomers as monitored by circular dichroism (data not shown) and analytical ultracentrifugation (see below) at low temperatures. However, they all have less negative circular dichroism signals at low temperature than the MNIII mutant, suggesting that they might be partially unfolded even at low temperature.

The thermal unfolding of the NIII₆₋₃₃, MG_{d5}NIII, and MA_{d5}NIII peptides was performed at an identical total peptide concentration (Fig. 5). None of the unfolding curves for NIII₆₋₃₃, MG_{d5}NIII, or MA_{d5}NIII are visibly three state; they all show a single unfolding transition, consistent with a two-state behavior. Furthermore, the unfolding transitions of NIII₆₋₃₃, MG_{d5}NIII, and the MA_{d5}NIII peptides all can be superimposed on the second unfolding transition of the MNIII peptide. This suggests that the truncated and mutated variants of MNIII are thermodynamically and structurally similar to the folding intermediate of the MNIII peptide.

The oligomerization state of the MNIII variant

As mentioned above, the folding intermediate of MNIII is clearly not dimeric. Unfortunately, it is difficult to determine the oligomerization states of partially unfolded inter-

Fig. 5. Thermal denaturation of different variants in 10 mM phosphate buffer. MNIII (circles), NIII₆₋₃₃ (squares), MA_{d5}NIII (diamonds), and MG_{d5}NIII (×). The peptide concentration is 10 μ M for all peptides in terms of monomers.

Fig. 6. Sedimentation equilibrium analysis of NIII₆₋₃₃. The data were collected at 4°C, 200 μ M peptide. The distribution of the residuals is from the data fitting to a dimer-trimer equilibrium model. The expected slope for the dimer species is indicated by the thick black line. The Supplemental Material details the results of fitting various models to the data presented here for NIII₆₋₃₃.

mediates that are maximally populated at temperatures as high as 60°C (Fig. 2). To solve this problem, we examined the oligomerization of NIII₆₋₃₃, MG_{d5}NIII, and the MA_{d5}NIII peptides. We determined the apparent molecular weights of all the variants by sedimentation equilibrium at 4°C (Table 2). In each case, the data are better fitted by dimer-trimer or dimer-tetramer equilibrium models than by the other possibilities tested (Fig. 6; Supplemental Material).

Discussion

Stated simply, we have shown that moving a single Asn from one *a* position to another *a* position changes the folding pathway of a simple leucine zipper protein. The thermal unfolding of the MNIII variant can be described by a model shown in Figure 7. At very low temperatures, MNIII forms a stable, dimeric leucine zipper. As the temperature in-

Fig. 7. A model for the thermal unfolding pathway of MNIII with N₂ as a native dimer, I₂ as a partially unfolded intermediate dimer, I_{≥3} as a partially unfolded intermediate oligomer, and U as a fully unfolded monomer. Cylinders represent folded helices, whereas boldface curved lines represent unfolded random coils.

creases, the dimer of the MNIII peptide starts fraying at the N terminus and becomes partially unfolded. The localization of this fraying is presumably caused by the weaker α - α' interaction between pairs of asparagines than pairs of isoleucines (Zhu et al. 2000). This partially unfolded intermediate no longer has the same specificity to form dimers, and some of the molecules rearrange to form partially unfolded trimers. An equilibrium between partially folded dimers and trimers is established at temperatures in which the intermediate is populated, between 50°C and 70°C.

As the temperature increases further, the C-terminal regions of the peptides unfold, and the equilibrium is shifted to fully unfolded monomers. The change in oligomerization state is supported by the concentration dependence of the thermal denaturation and the change in molecular weight with the increase in temperature. We cannot exclude the possibility that the partially unfolded MNIII variant forms other higher-order oligomers than trimers at higher peptide concentrations, but a model that includes a mixture of dimers and trimers was able to fit the observed centrifugation data.

The fraying of the N terminus is consistent with the results from the limited proteinase K digestion at the temperature at which the intermediate is populated and also by the similarity of the thermal unfolding of NIII₆₋₃₃, MG_{d5}NIII, and MA_{d5}NIII to that of the second transition of MNIII. The helix contents of NIII₆₋₃₃, MG_{d5}NIII, and MA_{d5}NIII are lower than that of the MNIII peptide at low temperature, again suggesting a difference in fraying of the N terminus between various peptides.

The ability of MNIII to populate a stable, partially folded intermediate is probably related to the minimum length of a stable coiled coil. Even when the N-terminal heptad of MNIII is frayed, the remaining heptads retain enough interaction energy to form a cooperative folding unit. As we show here for NIII₆₋₃₃, peptides containing only three heptads derived from the GCN4 leucine zipper have been shown to form moderately stable dimers (Lumb et al. 1994; Su et al. 1994) and other model peptides form coiled coils with as few as two heptads (Kammerer et al. 1998; Steinmetz et al. 1998).

In contrast, the weakest α - α' interaction in the MINII and wild-type dimers is localized at the center of the dimer. When the coiled-coil structure around the central Asn pair denatures, neither side of the remaining structure is long enough to remain stably folded. Thus, the unfolding of the MINII and wild-type GCN4 peptides is cooperative and two state (Thompson et al. 1993; Qian 1994).

In this regard, partial unfolding of the MNII leucine zipper resembles multistate unfolding of other globular proteins (Jasanoff et al. 1994; Yang et al. 1997; Zitzewitz et al. 1999). The N-terminal heptad, which is not stable enough to fold on its own, forms a subdomain that can unfold independently of the rest of the oligomer. Other long coiled

coils, such as tropomyosin, also show multistate unfolding (see, for example, O'Brien et al. 1996). This kind of unfolding is similar to the partial denaturation that is observed for another well-known double-helical biopolymer. Although DNA denaturation and renaturation are highly cooperative, conditions can be found in which weakly interacting segments can form partially denatured bubbles or forks in the double helix (Inman 1966). As is the case with renaturation of DNA, cooperative refolding of coiled coils involves a bimolecular nucleation step followed by a "zippering up" of the helices (Krylov et al. 1995). Recent kinetic refolding studies have shown that the nucleation occurs preferentially in the C-terminal heptads of the GCN4 leucine zipper (Zitzewitz et al. 2000).

The other surprise in the unfolding behavior of MNIII is the loss of specificity for dimer formation in the intermediate. Although the Asn residues destabilize the packing in the hydrophobic interface (Zhu et al. 2000), our results are consistent with other studies showing that the presence of the Asn residues is important for a coiled coil to maintain specificity for dimer formation. When the N-terminal heptad is folded at low temperature, the Asn residues are buried in the hydrophobic core such that an interhelical buried hydrogen bond is able to form between the two Asn residues (O'Shea et al. 1991).

The formation of the buried hydrogen bond is a key determinant of dimerization specificity and favors the formation of a dimeric coiled-coil structure. However, when the N-terminal heptad is unfolded at higher temperatures, the interstrand hydrogen bond between the two Asn residues might break. The loss of the buried hydrogen bond between the Asn residues could cause the peptide to lose oligomerization specificity, resulting in a dimer-trimer mixture of our N-terminally unfolded intermediates. However, MIIII, a peptide with Ile residues at all four α positions and no buried Asn residue, is predominantly dimeric (Harbury et al. 1994; Zhu et al. 2000), despite the identity of the last three heptads in MIIII and MNIII. Thus, the absence of the interstrand H-bond is not sufficient to predict a loss of specificity with respect to oligomeric order. The length of a coiled coil seems to be another factor that affects its preferred oligomerization state as well as its stability.

There are many GCN4 leucine zipper mutants known to form a trimers or a mixture of dimers and trimers, when the buried Asn residue is mutated to Ala, Gln, or Val (Gonzalez et al. 1996a, 1996b). The identity of the α position also affects the oligomerization state of other model coiled coils (Wagschal et al. 1999a, 1999b). However, note that although the MNIII intermediate, NIII₆₋₃₃, MG_{d5}NIII, and MA_{d5}NIII are mixtures of dimers and trimers, the MNIII native state is predominantly dimeric.

Several groups have described programs for predicting coiled-coil structures from sequence analysis (Lupas et al. 1991; Berger et al. 1995; Woolfson and Alber 1995; Lupas

1996; Wolf et al. 1997). MULTICOIL (Wolf et al. 1997) correctly predicts that MNIII and MINII should be dimeric (dimer probabilities of 0.921 and 0.881, respectively, at position 16). However, it gives trimer probabilities in the order MNIII (0.084) < MA_{d5}NIII (0.107) < MINII (0.113), and it also predicts that the MA_{d5}NIII coiled coil extends through the first heptad. MULTICOIL predicts that NIII₆₋₃₃ and MG_{d5}NIII will not form coiled coils at all ($P = 0.075$ and 0.152 , respectively, compared to >0.88 for all of the others). The window averaging algorithms used by all of these programs may not take into account the length and heptad order effects that seem to underlie our experimental observations. In addition, note that the kinds of oligomers we observe are highly dependent on both the peptide concentration and the temperature. The differences in the expected concentration dependence for formation of dimeric versus higher-order oligomers could mean that predicting oligomerization states from sequence data alone may be inherently unreliable. Although our peptide forms a mixture of dimers and trimers, it may be possible to find peptides that undergo a more dramatic switch in oligomerization state as a function of concentration and temperature.

In conclusion, we have examined the unfolding of two leucine zipper peptides that differ only by the exchange of two residues at equivalent heptad positions. Although both leucine zippers are dimeric in the native state and the differences between their sequences are small, their unfolding pathways are dramatically different. These results highlight the difficulties in understanding the complexities of the protein folding and design processes.

Materials and methods

Strains and media

The variants MNIII and MINII were subcloned from pXZ series plasmids that encode λ cI-GCN4 leucine zipper fusion proteins (Zeng et al. 1997) into pZH3 peptide expression vectors, and transformed into BL21(DE3) as described previously (Zhu et al. 2000); MG_{d5}NIII and MA_{d5}NIII were constructed from MNIII by PCR mutagenesis at *d* position 5 and expressed in BL21(DE3).

Peptide design and purification

The recombinant GCN4 mutant peptides were prepared as described previously (Zhu et al. 2000). NIII₆₋₃₃ was obtained from the limited proteinase K digestion of MNIII and purified by reverse phase HPLC (Resource RPC; Pharmacia). All peptides were stored as lyophilized powders at -20°C and dissolved in and dialyzed extensively against the appropriate buffer immediately before use. Peptide concentrations were determined by tyrosine absorbance in 6 M guanidine hydrochloride using an extinction coefficient of $1455\text{ M}^{-1}\text{ cm}^{-1}$ at 275 nm (Pace et al. 1995).

Measurement of the thermodynamic stability of the *a* position variants

Circular dichroism spectra were acquired on an Aviv 62DS spectropolarimeter equipped with a temperature control and stirring

unit. Samples were prepared in 10 mM potassium phosphate buffer, pH 7.0. The pH of the samples was checked immediately before each experiment. The denaturation of peptides was measured by monitoring the ellipticity change at 222 or 234.5 nm as a function of either temperature or urea. The thermal denaturation was performed as previously described (Zhu et al. 2000). For the urea denaturation, experiments were performed at a peptide concentration 20- μM , at 25°C in 2.0 mL constant volume. The urea concentration in the cuvettes was increased from 0 M to 10 M in steps of 0.2–0.25 M with an equilibration time of 5.0 min and a data collection time of 99 sec. The urea solutions were freshly prepared daily in 10 mM potassium phosphate (pH 7.0). The concentration of the urea stock solution was determined by refractometry (Pace 1986). In all cases, both thermal and urea denaturation was completely reversible.

Limited proteolysis

Purified peptides were dissolved in and dialyzed against 50 mM phosphate buffer (pH 7.0) containing 150 mM NaCl. A 1.0-mM solution of each peptide was subjected to proteinase K (Sigma) digestion at 45°C for 10 hr, with a ratio of peptides to proteinase K of 10^4 :1 (w/w). The completion of the reaction was monitored by MALDI-TOF mass spectrometry. The reaction was quenched by addition of PMSF to a final concentration of 4 mM. The proteolytic product was purified by reverse phase HPLC (Resource RPC; Pharmacia), lyophilized and stored at -20°C as a solid powder. N-terminal sequencing was performed by automated Edman degradation at the Protein Chemistry Laboratory at Texas A&M University.

Determination of the oligomerization states by sedimentation equilibrium

Sedimentation equilibrium was performed as previously described (Zhu et al. 2000). Experiments were performed at either 4 or 37°C . Absorbance measurements at 288 nm, 275 nm and 225 nm were used to monitor the concentration distribution of the peptides at 1.0 mM, 200 μM and 40 μM , respectively.

Data analysis

For a dimeric two-state unfolding transition, the equilibrium between native dimer (N_2) and unfolded monomers ($2U$) is

where $K_d = [U]^2/[N_2]$. Given that the total peptide concentration, P_t , in terms of monomers is $P_t = 2[N_2] + [U]$, the CD signal Y_0 , can be described in terms of native and unfolded baselines, Y_N and Y_U , respectively, by the expression (Zhu et al., 2000):

$$Y_0 = (Y_U - Y_N) \frac{\sqrt{K_d^2 + 8K_dP_t} - K_d}{4P_t} + Y_N \quad (2)$$

where Y_N and Y_U were found to be constant at different urea concentrations. Given the dissociation constant

$$K_d = \exp(-\Delta G^0/RT) \quad (3)$$

and assuming that the apparent free energy difference between native and unfolded state, ΔG^0 , is linearly dependent on the urea concentration (Pace, 1986):

$$\Delta G^0 = \Delta G_{H_2O}^0 - m[Urea] \quad (4)$$

where $\Delta G_{H_2O}^0$ is the free energy difference in the absence of denaturant and m is the dependency of the unfolding transition to urea (Becktel and Schellman 1987; Matthews 1987; Pace 1986). By combining equations 2, 3, and 4, a single comprehensive equation, which describes the relationship between Y_0 and $[urea]$, was used to fit the equilibrium unfolding data sets. A nonlinear least-squares fit was performed in Kaleidagraph to obtain the $\Delta G_{H_2O}^0$ and m parameters for individual mutants. Global values for Y_N and Y_U were used for the different variants as described previously (Zhu et al. 2000).

MULTICOIL predictions were performed by submitting the peptide sequences to the MULTICOIL web page (<http://gaiberg.wi.mit.edu/cgi-bin/multicoil.pl>) at MIT.

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