Protein Science (1995), *4*:1934–1938. Cambridge University Press. Printed in the USA.

FOR THE RECORD

A calorimetric characterization of the salt dependence of the stability of the GCN4 leucine zipper

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(RECEIVED May 9, 1995; ACCEPTED July 18, 1995)

Abstract: The effects of different salts (LiCl, NaCl, ChoCl, KF, KCl, and KBr) on the structural stability of a 33-residue peptide corresponding to the leucine zipper region of GCN4 have been studied by high-sensitivity differential scanning calorimetry. These experiments have allowed an estimation of the salt dependence of the thermodynamic parameters that define the stability of the coiled coil. Independent of the nature of the salt, a destabilization of the coiled coil is always observed upon increasing salt concentration up to a maximum of ~0.5 M, depending on the specific cation or anion. At higher salt concentrations, this effect is reversed and a stabilization of the leucine zipper is observed. The effect of salt concentration is primarily entropic, judging from the lack of a significant salt dependence of the transition enthalpy. The salt dependence of the stability of the peptide is complex, suggesting the presence of specific salt effects at high salt concentrations in addition to the nonspecific electrostatic effects that are prevalent at lower salt concentrations. The data is consistent with the existence of specific interactions between anions and peptide with an affinity that follows a reverse size order ($F^- > Cl^- > Br^-$). Under all conditions studied, the coiled coil undergoes reversible thermal unfolding that can be well represented by a reaction of the form $N_2 \leftrightarrow 2U$, indicating that the unfolding is a two-state process in which the helices are only stable when they are in the coiled coil conformation.

Keywords: calorimetry; folding thermodynamics; leucine zipper; salt effects

The structural stability of the leucine zipper region of GCN4 has been measured spectroscopically (O'Shea et al., 1989; Krylov et al., 1994) and by high-sensitivity differential scanning calorimetry (Thompson et al., 1993). These studies have made it clear that hydrophobic interactions along the dimer interface play a crucial role in the high stability of the molecule and that electrostatic interactions may also play a role in the stability of the GCN4 leucine zipper (O'Shea et al., 1991; Thompson et al., 1993; Krylov et al., 1994). Several interhelical and intrahelical electrostatic interactions are revealed by the crystal structure of GCN4 (O'Shea et al., 1991). The distances between the pairs Lys 15 and Glu 20′, Glu 22 and Lys 27′, and Lys 27 and Glu 22′ suggest the existence of interhelical electrostatic interactions. In addition, intrahelical interactions are also apparent between Lys 8 and Glu 11 and between Glu 22 and Arg 25. According to the crystallographic structure, stabilizing interactions between oppositely charged ion pairs appear to be greater than destabilizing interactions between like charged residues, thus contributing to the overall stability of the folded dimer (O'Shea et al., 1989; Lumb et al., 1994). Recently, however, Lumb and Kim (1995) have observed that the charge–charge interaction between Lys 15 and Glu 20′ might be actually destabilizing at the salt condition of their studies (150 mM NaCl), judging by the observed stabilization after replacing the Glu residue by Gln.

The existence of stabilizing or destabilizing electrostatic effects can be evaluated by measuring the salt dependence of the stability of the coiled coil. In this paper, the dependence of the thermodynamic parameters on the concentration of different salts was investigated by high-sensitivity differential scanning calorimetry. Our results indicate that for all salts studied the effect of increasing salt concentration from 0 to 1 M is complex. The transition temperature initially decreases with increasing salt concentration, but eventually that trend is reversed. The salt concentration at which stabilization initially occurs and the extent of stabilization depend on the nature of the salt. These results indicate the presence of both stabilizing and destabilizing interactions, the predominance of which depends on the concentration and nature of the salt present in the solution.

Results and discussion

Differential scanning calorimetry

Two different series of high-sensitivity calorimetric experiments of the GCN4-33 leucine zipper peptide were performed at salt concentrations ranging from 0 to 1 M and identical concentra-

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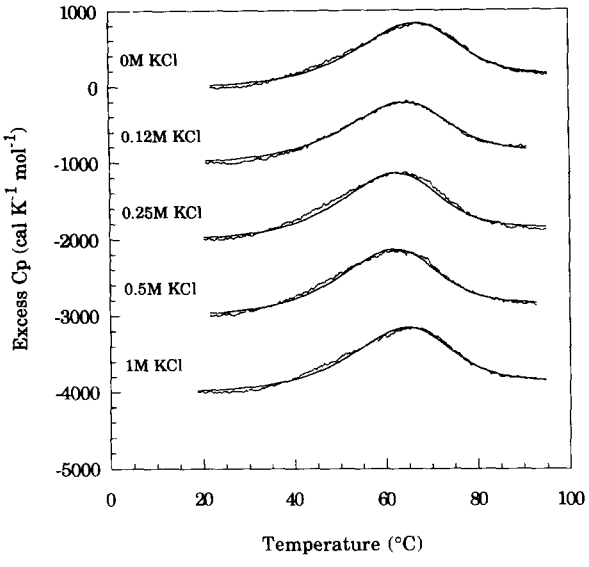

Fig. 1. Excess heat capacity of GCN4-33 leucine zipper as a function of [KCl]. Experiments were performed in 10 mM ACES, 0.25 mM EDTA, 0–1 M KCl, pH 7.0. The location of the temperature denaturation peak decreases as a function of salt concentration up to 400 mM, and then increases at higher concentrations. For ease of presentation, the data sets have been offset by 1 kcal/K·mol. In all cases, the smooth curves represent fits to the heat capacity data using using a two-state dimeric model as described earlier (Thompson et al., 1993).

tions of the peptide. One series of experiments was performed using salts with Cl^- as a common anion and differing cations: Li^+, Na^+, K^+, and $HOCH_2CH_2N{+}(CH_3)_3$ (choline) (10 mM ACES, 0.25 mM EDTA, 0–1 M XCl, pH 7.0). A second series was conducted using salts with K^+ as a common cation and differing anions: F^-, Cl^-, and Br^- and similar buffer conditions (10 mM ACES, 0.25 mM EDTA, 0–1 M KX, pH 7.0).

Figure 1 summarizes the results of the experiments performed at increasing KCl concentrations. In this figure, representative heat capacity curves have been plotted as a function of temperature. As shown in the figure, the location of the temperature denaturation peak first decreases upon increasing salt concentrations and then increases at concentrations higher than 0.4 M. Similar effects were observed for other salts as shown in Figure 2, where the temperature location of the maximum in the heat capacity function (T_m) has been plotted as a function of concentration for both the anion (Fig. 2A) and cation series (Fig. 2B). Identical conclusions are obtained if the temperature of half completion of the transition is plotted as a function of salt concentration. For the series of experiments with Cl^- as a common anion, LiCl destabilizes the peptide most, decreasing the T_m from 67.2 °C to 59.4 °C in the range 0–750 mM and then increasing the T_m to 60.2 °C at 1 M LiCl. The effects of NaCl and KCl are similar, decreasing the T_m by ~5 °C to 62 °C from 0 to 500 mM salt, followed by an increase in T_m to an average value of 64.5 ± 0.2 °C when the salt concentration is increased to 1 M. Choline chloride (ChoCl) destabilizes the peptide to a slightly greater extent in the 0–500 mM range from 67.2 °C to 61.2 °C, and stabilizes the T_m to only 62.6 °C from 500 mM to 1 M. For the series of experiments with a common cation (K^+), the effect is also complex, exhibiting an initial destabilization followed by a stabilization of the GCN4-33 leucine zipper peptide. The stabilization is more pronounced in the case of KF, where the T_m reaches 92 °C at 2 M.

In all cases considered, the thermal unfolding was well approximated by a two-state dimeric model of the type:

$$N_2 \leftrightarrow 2U, \qquad (1)$$

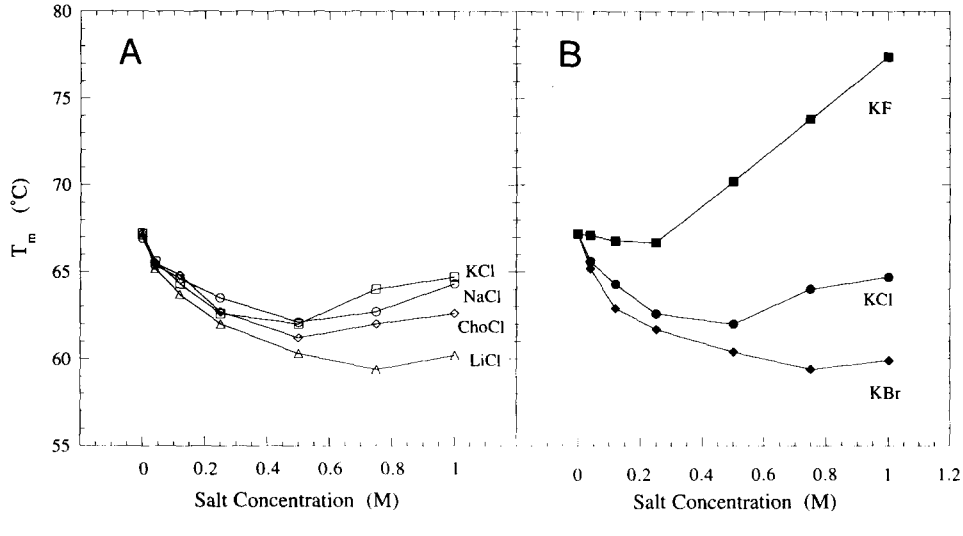

Fig. 2. A: Transition temperature of GCN4-33 leucine zipper unfolding as a function of salt concentration in 10 mM ACES, 0.25 mM EDTA, 0–1 M salt, pH 7.0, using salts with a common anion and differing cations. The salts shown are LiCl (△), NaCl (○), KCl (□), and ChoCl (◇). **B:** Transition temperature of GCN4-33 leucine zipper unfolding as a function of salt concentration in 10 mM ACES, 0.25 mM EDTA, 0–1 M salt, pH 7.0, using salts with a common cation and differing anions. The salts shown are KF, KCl, and KBr. Lines connect the points in order to clarify data sets and represent no attempt at fitting the data to any particular model. Peptide concentrations are constant within ±0.5% throughout all data sets in order to minimize the effect of peptide concentration on T_m.

in which the only states that become significantly populated during the transition are the folded dimer and the unfolded monomers. The solid lines in Figure 1 correspond to the best fit using this model. The dimeric nature of the folded form was checked by analytical centrifugation (see the Materials and methods). The two-state character was also checked by CD experiments as described earlier (Thompson et al., 1993; Kenar, 1994). Inclusion of intermediates in the analysis of the heat capacity function as described before for a longer GCN4 peptide (Thompson et al., 1993) did not improve the goodness of the fit. The two-state character of the thermal unfolding of the GCN4 leucine zipper has been verified independently by unfolding and refolding kinetic studies (C.R. Matthews, pers. comm.). Only at very high KF concentrations (>1.5 M) does a deviation from the two-state behavior occur, probably due to the stabilization of isolated helices at low temperatures (Kenar, 1994).

According to the two-state dimeric model, at any temperature and peptide concentration, $[P_T]$, the population of molecules in the monomeric unfolded state, P_U, can be expressed by the equation:

$$P_U = K \cdot [(K^2 + 4)^{1/2} - K)]/2, \quad (2)$$

where

$$K = \exp(-\Delta G/RT)/(2 \cdot [P_T])^{1/2}.$$

In this case, the temperature of the maximum in the heat capacity function does not coincide with the temperature at which half of the molecules are unfolded. It can be shown rigorously that, for the two-state dimeric model, the maximum in the excess heat capacity function occurs at the temperature in which $P_U = (2 - 2^{1/2})$ (Freire, 1989). Rearrangement and evaluation of the above equation at the temperature at which the heat capacity is maximal, T_m, yields the following expression for the entropy change:

$$\Delta S(T_m) = \Delta H(T_m)/T_m + R \cdot \{0.2525 + 1/2\ln([P_T])\} \quad (3)$$

where the enthalpy, $\Delta H(T_m)$, entropy, $\Delta S(T_m)$, and protein concentration, $[P_T]$, are expressed on a per mole of monomer basis. In this paper, all thermodynamic parameters are expressed on a per mole of monomer basis.

Table 1 summarizes the experimental thermodynamic data obtained calorimetrically for the six different salts studied and evaluated at the transition temperature in the absence of extra salt (67.2). A plot of the enthalpy values presented in Table 1 as a function of temperature yields a straight line with a slope (ΔC_p) equal to 250 ± 20 cal/K·mol. This value is similar to the one obtained from the individual heat capacity curves. At 60 °C, the enthalpy change averages 21.4 ± 0.9 kcal/mol independently of the salt. The enthalpy change for the transition does not exhibit a measurable dependence on the nature of the salt. These observations indicate that the salt effect on the stability of the peptide is primarily entropic, as observed for other proteins.

Figure 3 displays the change in the Gibbs free energy of stabilization of the peptide elicited by the various salts. As shown in the figure, the destabilizing effect levels off around 0.5 M, with a magnitude on the order of 400 cal/mol, depending on the specific anion or cation. At higher salt concentrations, the stabilizing effects begin to predominate as manifested by the change in the slope of the curves, especially for KF. Because salt concentration per se does not significantly affect the enthalpy or heat capacity changes, the magnitude of its stabilizing/destabilizing contribution to ΔG is expected to be independent of temperature to a large extent. At low salt concentration, the overall Gibbs free energy of stabilization at 25 °C is 4,000 cal/mol, indicating that, except for KF, salt concentration can modulate the magnitude of the Gibbs free energy by about 10% at that temperature. In commonly used salts such as NaCl or KCl, the stability of the coiled coil is lowest between 0.3 and 0.5 M salt. Under these conditions, an increase or a decrease in salt concentration will stabilize the peptide.

Table 1. *Salt dependence of folding/unfolding thermodynamic parameters for the leucine zipper of GCN4*[a]

Salt	M	T_m	ΔH_{T_m}	ΔS_{T_m}
Ref		67.2	22.5	58.84
KCl	0.04	65.6	22.5	59.15
	0.12	64.3	21.6	56.74
	0.25	62.6	22.3	59.15
	0.50	62.0	22.3	59.27
	0.75	64.0	21.6	56.79
	1.00	64.7	22.3	58.73
NaCl	0.04	65.4	22.1	58.01
	0.12	64.6	23.0	60.83
	0.25	63.5	22.4	59.27
	0.50	62.1	22.3	59.25
	0.75	62.7	22.3	59.13
	1.00	64.3	22.9	60.59
ChoCl	0.04	65.5	22.0	57.69
	0.12	64.8	21.9	57.53
	0.25	62.7	22.5	59.72
	0.50	61.2	21.7	57.63
	0.75	62.0	22.5	59.86
	1.00	62.6	21.9	57.95
LiCl	0.04	65.2	22.1	58.04
	0.12	63.7	22.1	58.34
	0.25	62.0	21.5	56.88
	0.50	60.3	21.5	57.21
	0.75	59.4	21.1	56.18
	1.00	60.2	21.5	57.22
KF	0.04	67.1	22.3	58.27
	0.12	66.8	22.9	60.09
	0.25	66.7	22.9	60.11
	0.50	70.2	23.7	61.75
	0.75	73.8	24.4	63.05
	1.00	77.4	26.7	68.89
KBr	0.04	65.2	21.9	57.45
	0.12	62.9	22.7	60.28
	0.25	61.7	22.1	58.73
	0.50	60.4	21.5	57.19
	0.75	59.4	21.1	56.18
	1.00	59.9	21.4	56.98

[a] The reference condition is 10 mM ACES, 0.25 mM EDTA, pH 7.0. The temperature of the maximum in the heat capacity function (T_m) is given in °C. The enthalpy change at T_m (ΔH_{T_m}) is given in kcal/mol. The entropy change at T_m (ΔS_{T_m}) is given in cal/K·mol. The error in T_m is ±0.2 °C. The error in enthalpy determination is ±5% and is primarily due to uncertainties in baseline determination.

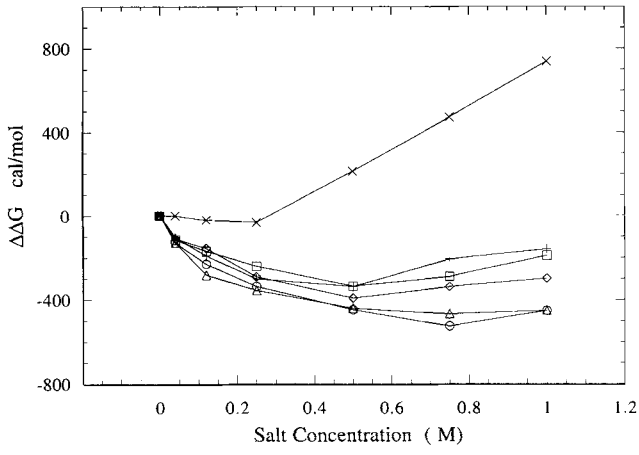

Fig. 3. Effect of different salts on the experimental free energy of stabilization of the GCN4-33 coiled coil. The $\Delta\Delta G$ values have been calculated at the transition temperature in the absence of added salt (67.2 °C). The salts shown are LiCl (○), NaCl (□), ChoCl (◇), KF (×), KCl (+), and KBr (△).

Analysis of the anion dependence

For KF, the T_m of the peptide decreases by 0.5 °C from 0 to 250 mM, and then increases from 66.7 °C to 77.4 °C as the concentration of KF is increased from 250 mM to 1 M. KCl destabilizes the peptide by 5.2 °C in the 0–500 mM range of salt concentration, and stabilizes it by only 2.7 °C up to 1 M KCl. KBr destabilizes the peptide even further, from 67.2 °C to 59.4 °C in the range 0–750 mM, and stabilizes it only slightly (0.5 °C) when the concentration is increased to 1 M.

The presence of stabilizing electrostatic interactions predicts a decrease in the stability of the peptide upon increasing ionic strength. This has been corroborated experimentally at low salt concentrations. However, in all cases, the stability of the peptide eventually begins to increase at higher salt concentrations. The magnitude of this increase depends on the nature of the ions. Nonspecific ionic strength effects, such as charge-charge shielding, predict destabilization throughout the entire concentration range and fail to explain the specific ion dependencies observed. Stabilization at the higher salt concentrations can be explained by a combination of specific ion effects. Because in this series of experiments the cation is the same, differences in the stabilization behavior of the various salts can be attributed to the nature of the anion. From a purely thermodynamic standpoint, the effect of an additive (salt, ligand, etc.) on the stability of a protein can be expressed in terms of the linkage equation (Wyman, 1964):

$$-\frac{1}{RT}\frac{\partial \Delta G}{\partial \ln X} = \Delta \upsilon \qquad (4)$$

where X is the activity of the additive and $\Delta\upsilon = \upsilon_U - \upsilon_N$ is the difference in the preferential interaction of the additive with the unfolded and native states. Addition of X will stabilize the state that interacts preferentially with the additive. For different salts, the difference in the interactions of the additive with the native and unfolded states reflects changes in the balance between salt exclusion and salt binding between those two states (Arakawa et al., 1990a, 1990b). For the series of experiments in which the cation is kept constant, stabilization of the peptide at higher salt concentrations can best be explained by postulating a specific interaction between anions and the peptide. Within this context, the existence of a stabilizing effect indicates that the interaction of an ion with the folded state of the peptide is of greater affinity than its interaction with the unfolded state. Ranking the anions in the order of their ability to stabilize the peptide reveals that they follow a definite size trend ($F^- > Cl^- > Br^-$), with the smallest anion (F^-) being most stabilizing. This order is identical to that seen by von Hippel and Wong (1965) in their studies on ribonuclease. The observed results with the GCN4 peptide are consistent with the existence of weak anion binding able to discriminate between anions of different size.

Analysis of cation dependence

The destabilization/stabilization behavior produced by changes in cation concentration can be analyzed in the same terms used to account for the anion-dependent stabilization of the peptide. Initial destabilization by all four salts can be explained in terms of ionic strength effects, i.e., shielding of stabilizing electrostatic interactions. ChoCl was included in this study to represent a cation that would presumably be too large to bind specifically to a site on the peptide surface. Thus, effects on the stability of the leucine zipper in the presence of ChoCl will be due primarily to ionic strength effects and the putative interactions with the Cl^- ion. The concentration at which stabilization begins, 500 mM ChoCl, is the same as the concentration of stabilization for KCl and NaCl, indicating that the loss of potential for cation binding is not substantially destabilizing. In general, the experiments with a common anion show a less pronounced cation dependence at higher salt concentrations, suggesting that specific cation binding events do not play a significant role.

Conclusions

These studies indicate that, at low ionic strength, the sum total of electrostatic interactions do in fact contribute favorably to the structural stability of the coiled coil leucine zipper. The nature of this effect is, however, complex as evidenced by the salt dependence of the stability of the peptide at neutral pH. Recently, Lumb and Kim (1995) have shown for the acetylated peptide that the replacement of Glu 20 by glutamine is slightly stabilizing (~0.15 kcal/mol). Taken together, these results indicate the existence of a delicate electrostatic balance resulting from competing stabilizing and destabilizing electrostatic interactions, and that small changes in the peptide or the environment are able to modulate the stability of the leucine zipper up or down. Overall, electrostatic interactions are only slightly stabilizing, indicating that not all salt bridges are stabilizing or that their influence is canceled out by the existing repulsive interactions between like charges. The complicated destabilization/stabilization effects observed by salt concentration apparently involve compensating effects including: (1) ionic strength effects, which are shown to be destabilizing as salt concentration increases and are nearly independent of the salt involved; and (2) anion-specific interactions with the folded peptide, which are stabilizing and dependent on the nature of the salt. Future studies should provide a quantitative evaluation of the contribution of each of these factors to the stability of the GCN4 leucine zipper.

Material and methods

The peptide was synthesized using the solid-phase technique (see Kent, 1988 for a review) on a Milligen model 9050 peptide synthesizer. Purity of the peptide was verified by reversed-phase HPLC on a Waters Delta Pak C18 column using a linear gradient of water/acetonitrile, both containing 0.1% TFA. Amino acid analysis was performed using the Waters Picotag system. The sequence of the peptide used in the experiments presented in this paper (GCN4-33) is:

NH_2-RMKQLEDKVEELLSKNYHLENEVARLKKLVGER-COOH.

ACES buffer, LiCl, and ChoCl were obtained from Sigma Chemical Company. KCl, KOH, NaCl, NaOH, and EDTA were obtained from J.T. Baker, Inc. KBr was obtained from Fisher Scientific. KF and LiOH were obtained from Aldrich Chemical Company. Spectra/por 3 dialysis membrane was purchased from Spectrum.

Sample preparation

For each salt series, buffers containing 10 mM ACES, 0.25 mM EDTA, and 0 M or 1 M of the appropriate salt were prepared volumetrically. Each of these buffers was then brought to pH 7.0 with the appropriate cation hydroxide (KOH, NaOH, or LiOH; KOH was used in the ChoCl series). Each solution contained 6–7 mM more cation than anion as a result of the addition of cation hydroxide. The peptide was dissolved in the appropriate buffer and dialyzed overnight through Spectra/por 3 dialysis membrane. Samples and buffers of intermediate salt concentration were prepared by dilution of the 0 M and 1 M solutions. Samples prepared for calorimetry were diluted with the appropriate buffer to an absorbance value of 1.005 ± 0.005 at 274.5 nm (4×10^{-4} M). Because the transition temperature of a system exhibiting monomer–dimer equilibrium is concentration dependent, extreme care was taken in order to ensure that all the experiments in the salt concentration series were performed at the same peptide concentration. It must be noted, however, that in the concentration range used in the calorimetric experiments, the dependence of the transition temperature on the peptide concentration is already in the plateau region (Thompson et al., 1993) and is not expected to change by more than 0.05 °C due to experimental uncertainties in concentration determination.

Differential scanning calorimetry

Samples were scanned at 60 °C h^{-1} in a DASM DS-92 microcalorimeter under a pressure of 17 psi. The calorimeter was interfaced to a PC microcomputer equipped with a Data Translation DT-2801 A/D converter board for instrument control and automatic data collection. The excess heat capacity function was analyzed after scan rate normalization, concentration normalization, and baseline subtraction using software developed in this laboratory for thermal transitions exhibiting dimer to monomer equilibrium, as described previously (Freire, 1989; Thompson et al., 1993). Under these conditions, the accuracy in the temperature determination is better than 0.1 °C. Individual baseline buffer scans were performed for each salt condition considered in this paper.

Analytical ultracentrifugation

Equilibrium sedimentation measurements were performed using a Beckman XL-A analytical ultracentrifuge equipped with absorbance optics and a Beckman An-60Ti rotor. Measurements were performed in 10 mM ACES, pH 7.0, 0.25 mM EDTA. Samples were loaded at three concentrations, 50 μM, 100 μM, and 200 μM into a six-hole centerpiece and spun at 40,000 rpm for 48 h. Twenty data sets collected at 215 nm for the three concentrations were averaged and jointly fit for a singular molecular weight. Compositional partial specific volumes for the proteins were calculated according to Zamyatnin (1984). The molecular weight estimated from these experiments was 7,640 Da. The molecular weight of the monomeric peptide calculated from the amino acid sequence is 3,994 Da. These experiments, as well as the observed concentration dependence of the thermal denaturation transition temperature, confirmed that the peptide is dimeric at low temperatures under the conditions of the experiments presented in this paper. CD measurements performed as described before (Thompson et al., 1993) (data not shown) also yielded the characteristic spectra of the coiled coil.

Acknowledgments

Supported by grants from the National Institutes of Health (RR04328) and the National Science Foundation (MCB-9118687). We thank Dr. Dmitry Krylov and Dr. Charles Vinson for performing the equilibrium centrifugation experiments and for many helpful discussions.

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