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The effect of N-terminal acetylation on the structure of an N-terminal tropomyosin peptide and αα-tropomyosin

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Abstract

We have used a synthetic peptide consisting of the first 30 residues of striated muscle α-tropomyosin, with GlyCys added to the C-terminus, to investigate the effect of N-terminal acetylation on the conformation and stability of the N-terminal domain of the coiled-coil protein. In aqueous buffers at low ionic strength, the reduced, unacetylated 32mer had a very low α-helical content (approximately 20%) that was only slightly increased by disulfide crosslinking or N-terminal acetylation. Addition of salt (>1 M) greatly increased the helical content of the peptide. The CD spectrum, the cooperativity of folding of the peptide, and sedimentation equilibrium ultracentrifugation studies showed that it formed a 2-chained coiled coil at high ionic strength. Disulfide crosslinking and N-terminal acetylation both greatly stabilized the coiled-coil α-helical conformation in high salt.

Addition of ethanol or trifluoroethanol to solutions of the peptide also increased its α-helical content. However, the CD spectra and unfolding behavior of the peptide showed no evidence of coiled-coil formation. In the presence of the organic solvents, N-terminal acetylation had very little effect on the conformation or stability of the peptide.

Our results indicate that N-terminal acetylation stabilizes coiled-coil formation in the peptide. The effect cannot be explained by interactions with the "helix-dipole" because the stabilization is observed at very high salt concentrations and is independent of pH. In contrast to the results with the peptide, N-terminal acetylation has only small effects on the overall stability of tropomyosin.

Keywords: circular dichroism; coiled-coil; helix-dipole; N-terminal acetylation; peptide folding; tropomyosin

Peptides are often used as models in protein conformation and stability studies (reviewed in Scholtz & Baldwin, 1992). Several investigators have shown that charged residues near the N- and C-termini of model peptides can have either negative or positive effects on the stability of isolated α-helices (Shoemaker et al., 1985, 1987a, 1987b; Marqusee & Baldwin, 1987). For example, peptides acetylated at the N-terminus appear to form more stable α-helices than their unacetylated analogues. It has been suggested that a positively charged group at the N-terminus of a small peptide may destabilize the α-helix through a repulsive electrostatic interaction with the helix dipole of the peptide (Fairman et al., 1989). Alternatively, it has been suggested (Quiocho et al., 1987) that esterification of the N-terminus may stabilize an α-helix by forming an additional hydrogen bond to a main-chain NH group. Such a bond cannot be formed by a free amino group.

Although factors that affect the structure and stability of coiled-coil model peptides have been investigated extensively (Hodges et al., 1972, 1981, 1990; Lau et al., 1984; Hu et al., 1990; O'Neil et al., 1990; Engel et al., 1991; Holtzer & Holtzer, 1992; Zhou et al., 1992a, 1992b), the effect of *N*-acetylation on the stability of coiled coils is unknown. We have been especially interested in the effects of N-terminal acetylation on the func-

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Abbreviations: TM32, 32-residue peptide having the sequence of rabbit striated muscle tropomyosin, residues 1-30, with GlyCys added at the C-terminus; AcTM32ox, N-terminal-acetylated TM32 peptide crosslinked by a disulfide to form a dimer; AcTM32re, reduced N-terminal-acetylated TM32 peptide; TM32ox, unacetylated TM32 peptide crosslinked by a disulfide to form a dimer; TM32re, unacetylated reduced TM32 peptide; DTT, dithiothreitol.

tion, structure, and stability of tropomyosin, a coiled-coil, actin-binding protein, found in muscle and nonmuscle cells (for reviews see Cohen & Parry, 1986, 1990; Lees-Miller & Helfman, 1991). Naturally occurring tropomyosin is N-terminally acetylated. In contrast, tropomyosin expressed in *Escherichia coli* has an unacetylated amino-terminus that would be protonated at physiological pH. Compared to acetylated vertebrate striated α-tropomyosin isolated from muscle, the unacetylated protein polymerizes poorly and has at least a 30-fold weaker affinity for actin (Hitchcock-DeGregori & Heald, 1987; Heald & Hitchcock-DeGregori, 1988).

The N-terminal 9 residues of tropomyosin are not currently resolved in the 9-Å tropomyosin structure (Phillips et al., 1986; Whitby et al., 1992). To study the effect of N-terminal acetylation on the conformation and stability of the N-terminal domain of tropomyosin, we designed a 32-residue peptide, TM32, based on the sequence of rabbit striated muscle α-tropomyosin (Stone & Smillie, 1978). We tested the effects of acetylation on the conformation and stability of this peptide and tropomyosin. *N*-acetylation greatly stabilizes the coiled-coil helical conformation of the dimeric 32mer, but only in the presence of high salt, suggesting that the stabilization is not due to an effect on the helix-dipole.

Results

Design of the TM32 peptide

We used a 32-residue peptide, TM32, to investigate the structure of the N-terminal region of tropomyosin and the effect of acetylation on its conformation and stability. This peptide consists of the first 30 residues of rabbit striated muscle α-tropomyosin (Stone & Smillie, 1978) with GlyCys added to the C-terminus. The cysteine residue was included at the C-terminus to allow disulfide formation because crosslinking stabilizes the α-helical conformation in coiled-coil peptides and proteins (Lehrer, 1978; O'Shea et al., 1989a; Hodges et al., 1990; Engel et al., 1991). The glycine residue was included to provide flexibility in the crosslinking region. In addition, the glycine residue places the cysteine at a *d* position in the coiled coil. Zhou et al. (1993) have shown that crosslinks introduced at this position stabilize coiled coils. The peptide was prepared both acetylated and unacetylated at the N-terminus. The complete sequence of the peptide is MDAIKKKMQMLKLDKENALDRAEQAEADKKGC. A model of the peptide, based on its homology with the GCN4 leucine zipper (O'Shea et al., 1991), is shown in Kinemage 1.

Effect of ionic strength on the conformation of the TM32 peptide

Short peptides with a high helical propensity are often disordered in near physiological buffers (Scholtz & Baldwin, 1992) and the TM32 peptide was no exception. At low to physiological ionic strength (0.01–0.15 N) the apparent total helical content of the reduced, unacetylated, N-terminal peptide of tropomyosin (TM32re) was approximately 20% at 2 °C. This low amount of helix was only slightly decreased by raising the temperature to 70 °C and disulfide crosslinking had no effect. N-terminal acetylation caused only small effects on the CD spectrum of the 32mer at low ionic strength. There were slight changes in the shapes of the CD curves of both the oxidized and reduced peptides suggesting a small increase in helical content to approximately 25–30% (Brahms & Brahms, 1980), but the magnitude of the ellipticity at 222 nm of the acetylated peptide was within experimental error of that of the unacetylated form.

Increasing the ionic strength increased the α-helical content of the TM32 peptide (Fig. 1), as has been found for other short coiled-coil peptides (Lau et al., 1984). In buffers containing high salt (>1 M NaCl) the helix at 2 °C (Fig. 2A) and stability (Fig. 2B) of the peptide were greatly increased by both N-terminal acetylation and disulfide crosslinking to form a dimer. The effects of oxidation and acetylation were synergistic. For example, in 2.5 M NaCl, 20 mM sodium phosphate, acetylation increased the average total helical content of the oxidized peptide at 2 °C by 93% ($P < 0.001$) but increased the average helical content of the reduced peptide by only 37% ($P < 0.005$). The estimated helical contents of the oxidized and reduced forms of the acetylated peptide (mean ± SD) were 64 ± 6% ($n = 8$) and 37 ± 4% ($n = 13$) and of the oxidized and reduced unacetylated peptide were 33 ± 5% ($n = 6$) and 27 ± 9% ($n = 10$), respectively.

Effect of pH on the conformation of the oxidized TM32 peptide

To learn if the stabilizing effect of N-terminal acetylation is a consequence of deprotonation of the α-amino group, we examined the CD of the TM32 peptide as a function of pH. At low ionic strength (0.1 M NaCl, 0.02 M phosphate) the CD of the unacetylated, oxidized peptide was almost independent of pH over a range of pH 4–11. In high salt (2.5 M NaCl, 0.02 M phosphate), the CD spectra of both the acetylated and unacetylated oxidized peptide were independent of pH over a range of pH 4–10. Above pH 10 there was an equal increase in the helical content of both the acetylated and unacetylated oxidized peptide of 15% of the total. These results suggest that the lack of a positive charge of the N-terminal amino group is not responsible for the increased stability of the acetylated, coiled-coil peptide. However, neutralization of the side chains of the lysines above pH 10 in the peptide appears to increase its total helical content.

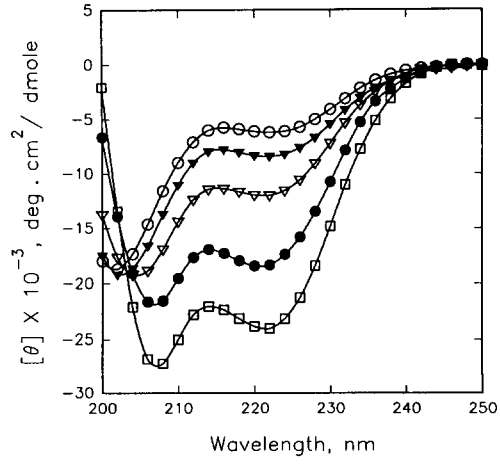

Fig. 1. Effect of increasing concentrations of NaCl on the CD spectra of AcTM32ox, 0.05 mg/mL, in 0.005 M sodium phosphate, pH 6.0 at 2 °C. ○, 0.025 M; ▼, 0.1 M; ▽, 1.3 M; ●, 2.5 M; □, 3.8 M.

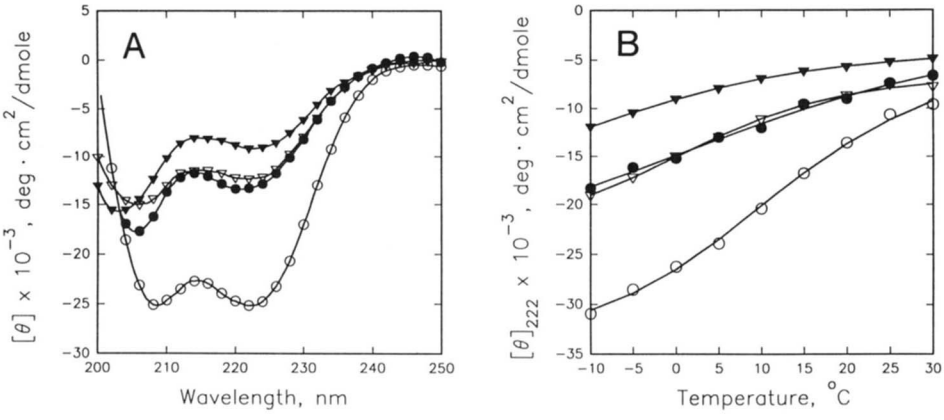

Fig. 2. Effect of oxidation and acetylation on the TM32 peptide, 0.05 mg/mL, in 0.02 M sodium phosphate, 2.5 M NaCl, pH 7.2. **A:** CD spectra at 2 °C. ○, AcTM32ox; ●, AcTm32re; ▽, TM32ox; ▼, TM32re. **B:** Mean residue ellipticity at 222 nm as a function of temperature. Note that the differences in the spectra in 2.5 M NaCl of AcTM32ox in Figures 1 and 2 are due to the difference in the concentration of phosphate.

Molecular weight of the acetylated 32mer in high salt

The molecular weights of the oxidized and reduced acetylated 32mer in 2.5 M NaCl were determined by sedimentation equilibrium centrifugation at 4 °C. The equilibrium molecular weight distributions (Fig. 3) indicated that the reduced form of the AcTM32 peptide was predominantly monomeric, whereas the oxidized form was predominantly a 2-chain species (i.e., 2 monomers crosslinked) in solution. In both cases there was a weak tendency to associate to higher order structures. It was not possible to determine if the larger species were dimers, trimers, or tetramers. The amounts of the higher molecular weight components of both the oxidized and reduced peptides were so small that attempts to distinguish between monomer–dimer and monomer–trimer or monomer–tetramer equilibria gave results that were within the standard deviations of the fit. In any case, the K_a's of dimerization were so low ($1\text{–}4 \times 10^2\ M^{-1}$) that the higher order structures would not be present at the concentrations used for the CD studies at 4 °C and higher.

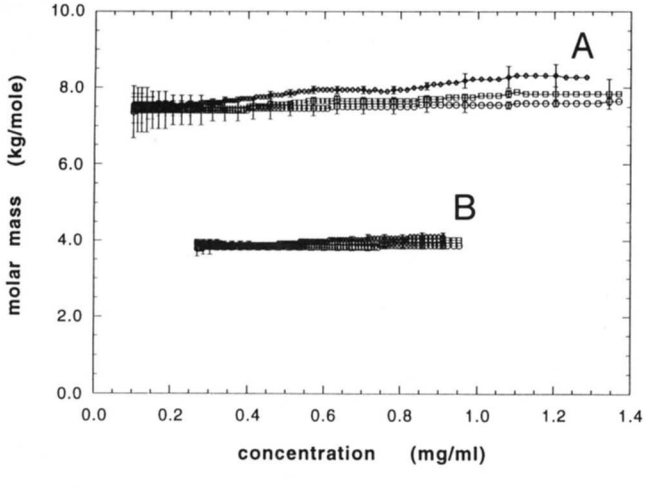

Fig. 3. Sedimentation equilibrium analytical ultracentrifugation of (**A**) AcTM32ox and (**B**) AcTM32re. The run was performed at 4 °C, 56,000 rpm. Buffer: 2.5 NaCl, 20 mM sodium phosphate, pH 7.2, without and with 1 mM DTT. The plot is of number (○), weight (□), and *z*-average molecular weight averages (◇) as a function of the local cell concentration at equilibrium. The calculated molecular weight of the monomer from its amino acid composition is 3,691.

Evidence that the TM32 peptide forms a coiled coil in 2.5 M NaCl

To learn the effects of acetylation on the structure of the peptide it is important to be able to distinguish the coiled-coil α-helix (Crick, 1952) (with a rise of 5.15 Å/turn, referred to as coiled coil) from the conventional α-helix (with a rise of 5.4 Å/turn) or other helical conformations, such as the 3_{10} helix, or helices in molten globules. Although the ellipticity at 222 nm is a measure of total helical content, it does not indicate the type of helix. Cooper and Woody (1990) calculated that the spectrum of a coiled-coil helix would be somewhat different from that of a single-stranded rigid helix. In the coiled coil, the ellipticity at 208 nm is decreased relative to that of the noncoiled helix, but the ellipticity at 222 nm is almost unchanged.

To determine whether the fully folded form of the TM32 peptide had a spectrum typical of a coiled coil, as opposed to a conventional α-helix, we examined the CD spectrum as a function of temperature. The results with AcTM32ox are shown in Figure 4A. Figure 4B shows that the degree of folding was identical when measured at 222 and 208 nm. To determine how many different conformational states contributed to the CD of the TM32 peptide, the spectra in Figure 4A were deconvoluted into 2, 3, and 4 basis curves using the convex constraint algorithm (Perczel et al., 1991, 1992). We have previously used this method to analyze the folding of tropomyosin and a 43-residue tropomyosin-like peptide, TM43 (Greenfield & Hitchcock-DeGregori, 1993). Figure 4C shows the components when the spectra in Figure 4A were resolved into 3 basis spectra. Only 2 component curves could clearly be distinguished. One component was similar to that of the coiled-coil α-helix present in native tropomyosin. As the peptide was cooled from 50 to −10 °C, the amount of coiled coil increased. The other 2 curves were almost identical and were similar to the spectrum of the peptide in low salt. Curve fitting using the method of Greenfield and Fasman (1969) would indicate that this spectrum was mainly random coil with a small amount of residual α-helix. Recently, however, Woody (1992) has suggested that such a spectrum, with a negative n–π* transition, is characteristic of disordered polypeptides in which strong free energy minima occur when the side chains are in the β and α_R regions of conformational space. The spectra of all 4 forms of the peptide in 2.5 M NaCl could be well fit by combinations of the 2-basis spectra. These results suggest that the folding of all 4 forms of the TM32 peptide represents a 2-state

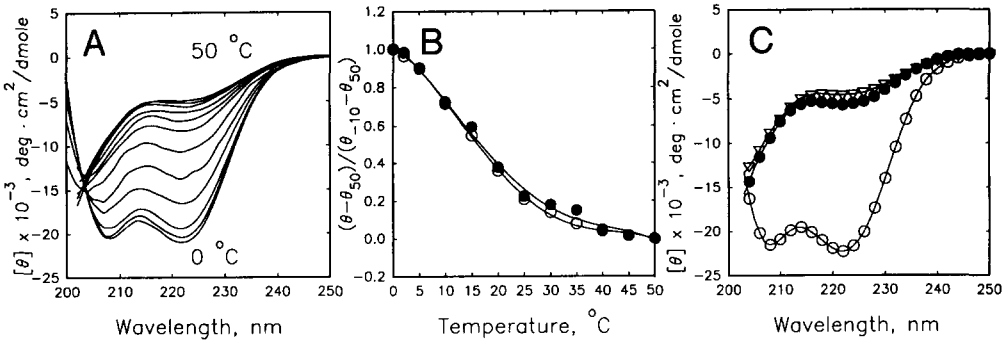

Fig. 4. A: Mean residue ellipticity of AcTM32ox, 0.05 mg/mL, in 2.5 M NaCl, 20 mM sodium phosphate, pH 7.2, as a function of temperature. **B:** Relative change in ellipticity at 222 (○) and 208 (●) nm of the data in Figure 6A between −10 and 50 °C. **C:** Basis spectra obtained when the data in Figure 5A were deconvoluted into 3 curves using the convex constraint algorithm of Perczel et al. (1992). ●, Coiled coil; ▽, ○, disordered.

cooperative transition between the disordered and coiled-coil conformations. In the absence of disulfide crosslinking, however, the amount of coiled coil formed is very low above 4 °C. Note that there could be a small amount of single-stranded α-helix contributing to the spectrum of AcTM32re above 4 °C because the CD at 222 nm decreases slightly as a function of temperature (Fig. 2B) but the ultracentrifugation results show the peptide to be monomeric. However, the amount of this helix would be too low to resolve as a unique spectrum using the convex constraint algorithm (Perczel et al., 1992).

The CD analysis, together with the sedimentation equilibrium results, indicates that the oxidized peptide is a parallel, 2-chained coiled coil at the concentrations used for the CD experiments (0.01–1.0 mg/mL). This peptide appears to undergo a 2-state helix–coil transition upon thermal denaturation. These findings are in contrast to results obtained with chicken striated muscle αα-tropomyosin and a related 43-residue peptide (Greenfield & Hitchcock-DeGregori, 1993). The folding of those molecules was not fully cooperative and deconvolution of their temperature-dependent spectra resolved a minimum of 3 basis curves and indicated the presence of a helical folding intermediate.

Thermal stability of the TM32 peptide in high salt

In order to ascertain if N-terminal acetylation affected the entropy or enthalpy of folding of the TM32 peptide we examined the thermal stability of the acetylated and unacetylated, oxidized and reduced peptides using CD (Fig. 2B). In 2.5 M NaCl the folding of both the acetylated and unacetylated disulfide-crosslinked peptides was cooperative. Acetylation increased the T_M of folding of the crosslinked peptide by 10 °C from 2 ± 3 °C ($n = 7$) to 13 ± 2 °C ($n = 8$), $P < 0.001$. The stabilization appeared to result from a small decrease in the Van't Hoff enthalpy of folding from −16 ± 2 to −22 ± 4 kcal/mol ($P < 0.01$). The increased stability cannot be explained by an entropy effect because when the peptide was acetylated the apparent entropy of folding actually decreased from −0.058 ± 0.006 kcal/mol·deg to −0.075 ± 0.015 kcal/mol·deg ($P < 0.01$). The same curves were obtained when the peptide was either heated from 2 to 70 °C or cooled from 70 to 2 °C, suggesting that the data were obtained under conditions of thermodynamic equilibrium. Moreover, the same thermodynamic parameters were obtained with data obtained between −10 and 30 °C and between 0 and 70 °C, showing that the data were sufficient to estimate the Van't Hoff enthalpy from the curves. The calculated T_M's agreed with the experimental T_M's. Although CD does not provide a direct measurement of enthalpy, Thompson et al. (1993) have recently shown that CD and differential scanning calorimetry measurements give identical values for the enthalpy of the helix–coil transition of the coiled-coil region of the GCN4 leucine zipper. This shows that determinations using CD can be valid for short 2-chained coiled-coil peptides, even when there is a small change in heat capacity upon unfolding.

The changes in ellipticity of the reduced peptides as a function of temperature were much less cooperative than those of the oxidized peptides (Fig. 2B). Unambiguous endpoints could not be determined, making it impossible to estimate the enthalpy of folding and the T_M's from the CD data. The folding of the reduced, unacetylated peptide was highly dependent on concentration, as might be expected for a monomer to dimer transition, although a transition from a monomer to a trimer or tetramer cannot be ruled out. Unfortunately, the association constant and order of the association were impossible to determine from the CD data, as the peptide was less than 50% folded at the highest concentration examined (0.6 mg/mL) at 2 °C.

The folding of AcTM32re, in contrast to that of TM32re, was almost independent of peptide concentration, between 2 and 70 °C, over a concentration range of 0.01–0.4 mg/mL, consistent with its being mainly monomeric under these conditions. The lack of concentration dependence of the spectrum of AcTM32re suggests that N-terminal acetylation increases the entropy barrier to self-association relative to the unacetylated peptide, consistent with the decrease in entropy of folding of AcTM32ox relative to TM32ox. At concentrations below 0.03 mg/mL, where both the acetylated and unacetylated peptides are essentially monomeric at 2 °C in 2.5 M NaCl, acetylation had only a small stabilizing effect on the apparent total helical content, increasing it from about 20 to 30%.

Homology modeling of the structure of the TM32 peptide

To give insight into the possible structural basis for the stabilization of the coiled coil by N-terminal acetylation, we modeled the acetylated oxidized TM32 peptide based on its homology

with the GCN4 leucine zipper (O'Shea et al., 1991). The N-terminal 9 residues are not currently resolved in the 9-Å tropomyosin structure (Phillips et al., 1986; Whitby et al., 1992). The residues of the TM32 peptide could easily be accommodated into the GCN4 structure, though this is, of course, not proof of the actual structure. As seen in Kinemage 1, the modeled structure of the TM32 peptide has many fewer large hydrophobic side chains in the *a* and *d* positions of the heptad repeat at the helix–helix interface than the GCN4 peptide, accounting for the lower stability of the TM32 peptide. The α-helices of the TM32 peptide may be stabilized by the observed *i* to *i* + 4 side-chain interactions of Asp-2 and Lys-6, Lys-12 and Glu-16, and Glu-26 and Lys-30 (1 less than in the GCN4 leucine zipper), and two *i* to *i* + 3 side-chain interactions of Asp-2 and Lys-5 and Glu-26 to Lys-29 (Kinemage 2) (same number as the GCN4 peptide). One would expect such stabilization to be minimal, however, at the high ionic strength necessary for coiled-coil formation. There is also an interchain interaction between *e* and *g* residues, Arg-21 and Glu-26.

The backbone structure of the modeled acetylated peptide was very similar to that of the unacetylated peptide. However, the N-terminal acetyl groups may be involved in several interactions not seen in the unacetylated form, which would stabilize the coiled coil. In the structure with the minimum calculated energy, the N-terminal ends of the 2 chains of the acetylated peptide were dissimilar, as in the parent GCN4 molecule. In the model, in chain 1, a hydrogen bond could be formed between the oxygen atom of the N-terminal acetyl group and the NH group of Ile-4 (Fig. 5A; Kinemage 3). In chain 2, the N-terminal acetyl group appeared to be relatively extended and could not form such an intrachain hydrogen bond. The methyl group of the N-terminal acetyl of chain 2, however, was very close (3.8 Å) to the side chain of Met-1 of peptide chain 1 (Fig. 5B; Kinemage 4) and could possibly participate in interchain hydrophobic interactions. In addition, there were possible interchain hydrophobic interactions between the acetyl group of chain 1 and the Met-1 side chain of peptide chain 2.

Effect of organic solvents on the ellipticity of the TM32 peptide

It is well known that the addition of alcohols to aqueous solutions of small peptides with helical propensity greatly increases their helical content (e.g., see Nelson & Kallenbach, 1989). Hodges et al. (1990) have suggested that coiled coils are not formed in such solvents. We investigated the effect of acetylation and oxidation of the TM32 peptide in 40% ethanol and 50% trifluoroethanol to determine the effects of these modifications of the α-helix, as opposed to the coiled coil.

Acetylation and disulfide crosslinking had no apparent effect on the helical content of the TM32 peptide in 40% ethanol (Fig. 6A) or 50% trifluoroethanol. The helical contents at 2 °C were approximately 60% in 40% ethanol and 65% in 50% trifluoroethanol for all 4 forms of the peptide. Moreover, oxidation and acetylation had no significant effect on the T_M values, or the slopes of the change in ellipticity as a function of temperature in the presence of either alcohol.

CD measurements indicated that the TM32 peptide did not form a coiled coil in solutions containing the alcohols, consistent with the chromatography results of Hodges et al. (1990). First, the folding of the oxidized peptide was less cooperative in either 40% ethanol or 50% trifluoroethanol than in 2.5 M NaCl (Fig. 6B). Mattice and Tilstra (1987) have calculated that the folding of interacting helices is more cooperative than the folding of single chains. Second, the ellipticity values were independent of peptide concentration in the presence of the alcohols. Finally, there was no evidence of a coiled-coil helical component in the CD spectra. The calculated Van't Hoff enthalpy, in buffers containing either 40% ethanol or 50% trifluoroethanol, was approximately 9 kcal/mol. This value is about half that in 2.5 M salt and reflects the lower cooperativity of the folding process.

Effects of N-terminal acetylation on the conformation and stability of $\alpha\alpha$-tropomyosin

To learn if acetylation affected the stability of full-length tropomyosin, we compared acetylated tropomyosin, isolated from striated muscle, and unacetylated tropomyosin, overexpressed in *E. coli*. Studies were performed in a buffer with near physiological ionic strength (100 mM NaCl, 20 mM phosphate, pH 7.2), and in a buffer with higher ionic strength (500 mM NaCl, 10 mM phosphate, 1 mM EDTA, 0.5 mM DTT, pH 7.5), which prevents self-association of the acetylated tropomyosins. There were similar effects of N-terminal acetylation on the ellipticity of intact tropomyosin as a function of temperature in both buffers. In

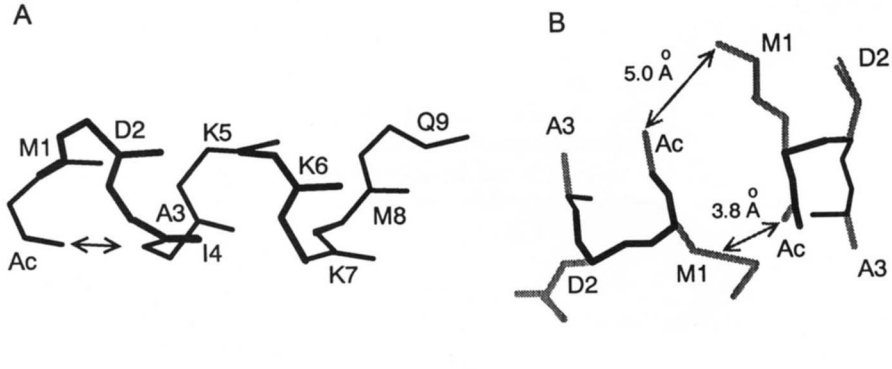

Fig. 5. Model of the structure of AcTM32ox based on that of the GCN4 DNA binding leucine zipper (O'Shea et al., 1991). **A:** Backbone bonds (CC, CN, and C=O) of the first 9 residues of chain 1. A possible hydrogen bond (arrow) between the carbonyl of the N-terminal acetyl group and the NH group of Ile-4 is illustrated. The amino acid residues are labeled at the carbonyl carbons. The NH bonds are not illustrated. **B:** First 3 residues of both chains of AcTM32ox, showing possible hydrophobic interactions between the side chains of Met-1 with the methyl groups of the N-terminal acetyl of the opposite chains. The backbone bonds (CC and CN) are in black. The side chains are in gray tone. The carbonyl groups and NH bonds are not illustrated.

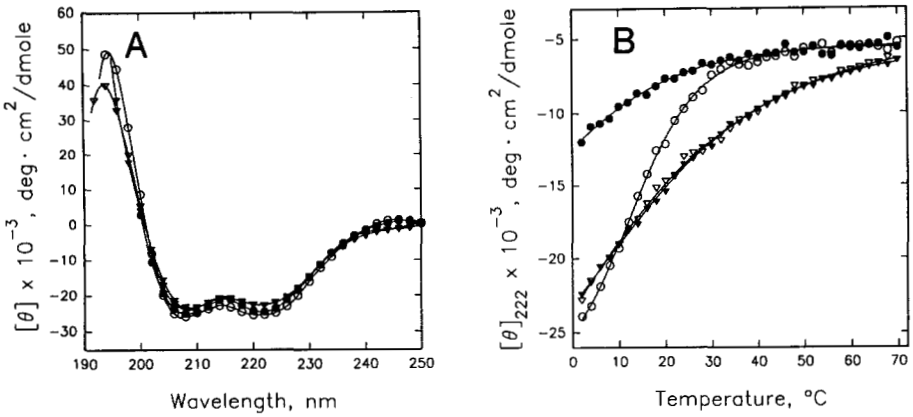

Fig. 6. A: Effect of acetylation and oxidation on the mean residue ellipticity of the TM32 peptide, 0.04 mg/mL, in 40% ethanol, 60% 20 mM sodium phosphate, 0.1 M NaCl, pH 7.1, at 2 °C. ○, AcTM32ox; ●, AcTM32re; ▽, TM32ox; ▼, TM32re. **B:** Effect of oxidation on the mean residue ellipticity the acetylated TM32 peptide, 0.075 mg/mL, as a function of temperature in buffer containing ethanol (40% ethanol, 60% 20 mM sodium phosphate, 0.1 M NaCl, pH 7.2) or high salt (2.5 M NaCl, 20 mM sodium phosphate, pH 7.2). ○, AcTM32ox in high salt; ●, AcTM32re in high salt; ▽, AcTM32ox in 40% ethanol; ▼, AcTM32re in 40% ethanol.

500 mM NaCl, acetylation increased the T_M of the cooperative unfolding transition of reduced chicken αα-tropomyosin approximately 2 °C. There was also a slight increase in the steepness of the unfolding transition, suggesting that N-terminal acetylation increases the enthalpy of unfolding (Fig. 7). In 100 mM NaCl, acetylation also increased the T_M of unfolding of chicken tropomyosin, approximately 2 °C for the reduced and 5 °C for the oxidized protein, and also increased the slope of the helix–coil transition. At the lower ionic strength, N-terminal acetylation had no effect on the T_M of unfolding of either oxidized or reduced rat/rabbit αα-tropomyosin, although it did increase the slope of the helix–coil transition of the reduced protein. (The sequences of rabbit and rat striated muscle tropomyosins are identical. Acetylated rabbit αα-tropomyosin was isolated from muscle; unacetylated rat tropomyosin was expressed in *E. coli*.) N-terminal acetylation had no effect on the ratio of the ellipticity at 222 to 208 nm as a function of temperature, and both the acetylated and unacetylated tropomyosins exhibited the same pretransitions. Similar results for the effects of acetylation on chicken αα-tropomyosin have been obtained by Ishii and Lehrer at the high ionic strength (unpubl. results).

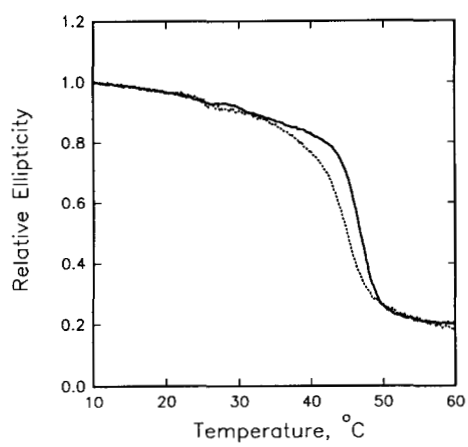

Fig. 7. Effect of acetylation on the ellipticity of αα-tropomyosin, 0.1 mg/mL, at 222 nm in 500 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, and 0.5 mM DTT, pH 7.1. ——, Acetylated chicken striated muscle αα-tropomyosin; ---, recombinant unacetylated chicken striated muscle αα-tropomyosin (expressed in *E. coli*).

Discussion

N-terminal acetylation is a common posttranslational modification in cytosolic proteins, yet its functional significance is generally unknown. In striated muscle αα-tropomyosin, however, N-terminal acetylation has been shown to be crucial for end-to-end association and for cooperative binding to filamentous actin (Hitchcock-DeGregori & Heald, 1987; Heald & Hitchcock-DeGregori, 1988). Here we have shown that N-terminal acetylation of an N-terminal peptide of tropomyosin stabilizes coiled-coil formation but has much smaller effects under conditions where the peptide does not form a coiled coil, i.e., in low salt, or at concentrations below 0.03 mg/mL in 2.5 M NaCl.

It has been suggested that N-terminal acetylation stabilizes α-helical peptides by removing a charge that opposes the helix-dipole (Wada, 1976; Hol et al., 1978; Hol, 1985). This mechanism probably does not account for the stabilization of the coiled coil by N-terminal acetylation observed in the TM32 peptide. First, stabilization was observed in the presence of high salt (2.5 M NaCl, 0.02 M sodium phosphate, pH 7.2). Fairman et al. (1989) have shown at such a high salt concentration the effects of N-terminal acetylation are minimized in their model monomeric helical peptides. Second, acetylation did not affect the stability of the peptide in 45–50% trifluoroethanol, although presumably the N-terminal amino group would be protonated in this solvent at neutral pH. Third, a stabilizing effect of acetylation was seen even above pH 10 where the N-terminal amino group would be uncharged.

Acetylation appeared to decrease the enthalpy of folding of the coiled-coil oxidized 32mer about 6 kcal/mol (approximately 3 kcal/N-terminus). The acetylated peptide has 1 more amide group than the unacetylated peptide. This group could participate in a hydrogen bond to the main chain (see Fig. 5A and Kinemage 3) and this may account for some of its increased stability, although the effect would be expected to be small. In addition, acetylation did not stabilize the protein in the presence of the alcohols, although such a hydrogen bond would be stabilized by the organic solvents.

Acetylation may possibly decrease repulsive interactions of the charged N-termini in the coiled coil. Such an explanation, however, would require that the ends are not shielded from each

other by the salts in the buffer. In addition, it would not explain the enhanced stability of the acetylated peptide relative to the unacetylated at very high pH.

The interesting question is what is the mechanism by which the effect of acetylation is so much more pronounced when the peptide is crosslinked and forms a coiled coil, than when it is reduced. The peptide model (Fig. 5B; Kinemage 4) suggests that the methyl groups of the acetyl side chains of each polypeptide chain could be involved in hydrophobic interactions with the side chains of the N-terminal methionines of the adjacent peptide chains. Such an interaction is possible because Met-1 in the TM32 peptide is in the *a* position and the side chains are in close proximity. This type of stabilizing interaction could only occur when the acetylated TM32 peptide forms a coiled coil and possibly could account for the synergistic effect of oxidation and acetylation. It has been shown that in the GCN4 leucine zipper, the β carbon of the side chain of Arg-1 of 1 chain, which is in a *g* position, has such hydrophobic interactions with the side chain of Met-2, which is in an *a* position, of the adjacent chain (O'Shea et al., 1991). The acetyl group of AcTM32 could substitute for the *g* position side chain. Such a mechanism would suggest that the position of an N-terminal acetyl group in the repeating heptad might dramatically affect its ability to stabilize a coiled coil. In the absence of a three-dimensional structure, such hypotheses, however, are pure speculation.

We have shown previously that in addition to N-terminal acetylation, the first 9 residues of tropomyosin are crucial for actin binding (Cho et al., 1990). One reason for designing TM32 was to determine if this region of tropomyosin is an actin-binding site. Acetylated, oxidized TM32 did not bind to actin, even at high peptide concentrations (0.9 mM). Neither the acetylated nor unacetylated oxidized peptide (1.4 mM) inhibited tropomyosin binding to actin. Unfortunately, in conditions for actin binding (50 mM NaCl, 10 mM Tris HCl, pH 7.5, 5 mM, $MgCl_2$, 5 μM F-actin, by cosedimentation) TM32 does not form a coiled coil.

Our results show that N-terminal acetylation does stabilize the coiled-coil α-helical conformation in a peptide, which consists of the N-terminal domain of tropomyosin. Acetylation may similarly stabilize the structure of the N-terminal end of intact tropomyosin, as suggested by the higher cooperativity of folding of the acetylated proteins, but the effects of such stabilization would be masked by its great length and high helical content.

Materials and methods

Peptide synthesis

The acetylated and unacetylated TM32 peptides were prepared at the protein synthesis facility at the Center for Advanced Biotechnology and Medicine, Piscataway, New Jersey, on an Applied Biosystems 430 peptide synthesizer by stepwise solid-phase procedures on a *t*-Boc-L-Cys(4-methylbenzyl)PAM resin. At the end of the synthesis, before cleavage from the resin, half of the peptide was acetylated using acetic anhydride. The peptides were purified by reverse-phase HPLC on a Vydac C18 column with a gradient of 0.1% trifluoroacetic acid, 10% H_2O to 0.1% trifluoroacetic acid, 10% H_2O, 20% isopropyl alcohol, 70% acetonitrile, and a flow rate of 6 mL/min. Mass spectrometric analysis of the purified peptides using fast atom bombardment at the CATF Mass Spectrometry Support Facility at Rutgers confirmed the anticipated masses for the acetylated and unacetylated peptides.

Sedimentation equilibrium measurements

Sedimentation equilibrium experiments to determine the molecular weights of the oxidized and reduced acetylated peptide were carried out on a Beckman Instruments Model-E Analytical Ultracentrifuge equipped with a digital real-time video-based data acquisition system and Rayleigh optics (Liu & Stafford, 1992). The video-based system automatically converts each digitized Rayleigh pattern into a computer disk file of fringe displacement versus radius. The camera lens was focused at the 2/3 plane of the cell. The cells were equipped with sapphire windows and 12-mm, 6-channel external loading centerpieces (Ansevin et al., 1970). Other details and methods of data analysis were as described previously (O'Shea et al., 1989b; Brenner et al., 1990). The peptides were in 2.5 M NaCl, 20 mM Na phosphate, pH 7.2, with or without 1 mM DTT.

Preparation of fully oxidized and reduced peptides

The peptide was completely reduced by incubating solutions with 1 mM DTT, or 5 mM 2-mercaptoethanol, for 10 min at 70 °C. The peptide was oxidized by incubating solutions, 10–20 mg/mL, in 100 mM NaCl, 20 mM sodium phosphate, pH 7.1, with 3-fold molar excesses of $K_3Fe(CN_6)$ and 0.3-fold molar additions of catalytic $CuSO_4$ as described by Ozeki et al. (1991). Following incubation at room temperature for 1 h, the reactions were stopped by adding 10-fold molar excesses of potassium EDTA, pH 8.0. The oxidized and reduced forms of the peptide were separated from one another and from components of the reaction mixtures by isocratic HPLC chromatography at room temperature (approximately 22 °C) on a Shodex Protein WS-8025 size exclusion column equilibrated with 0.1 M NaCl, 0.02 M sodium phosphate, pH 7.1, at 1 mL per min. The oxidized peptide eluted at 15.5 min and the reduced peptide at 16.2 min. The separated peaks each gave single peaks when rechromatographed, and they gave single bands upon SDS gel electrophoresis (Laemmli, 1970).

CD measurements

CD measurements were performed on an Aviv model 62 DS spectropolarimeter as previously described (Greenfield & Hitchcock-DeGregori, 1993). The protein content of peptide stock solutions was estimated by the micro-biuret procedure of Goa (1954). The total helical contents of the peptides were estimated from the ellipticity at 222 nm as described by Scholtz et al. (1991).

Curve fitting

The enthalpy and entropy of folding of the disulfide crosslinked peptides were estimated from changes in the CD spectra as a function of temperature using the equations:

$$k = \exp\{[\Delta H/(RT)]\,[(T/T_M) - 1]\} \quad (1)$$

and

$$\theta_{obs} = \{(\theta_{max} - \theta_{min})\,[k/(1 + k)]\} + \theta_{min}, \quad (2)$$

where θ_{obs} is the ellipticity found at any temperature, θ_{max} is the maximum ellipticity corresponding to the fully folded peptide, and θ_{min} is the ellipticity corresponding to the unfolded peptide. ΔH is the Van't Hoff enthalpy of folding, T is the absolute temperature, T_M is the midpoint of the folding transition, and R is the gas constant. θ_{max}, θ_{min}, T_M, and ΔH were estimated by curve fitting using the nonlinear least-squares routine supplied with the commercial program SigmaPlot 5.0 (Jandel Scientific). Similar results were obtained when θ_{max} was fixed at $-33{,}000$ deg·cm^2/dmol and θ_{min} was fixed at $-4{,}000$ deg·cm^2/dmol. These are the ellipticities of full-length folded chicken striated muscle $\alpha\alpha$-tropomyosin at 222 nm at 2 °C and unfolded at 70 °C, respectively (Greenfield & Hitchcock-DeGregori, 1993).

Preparation of tropomyosins

Striated muscle $\alpha\alpha$-tropomyosin was isolated and purified from chicken pectoral muscle or rabbit back and leg muscle as described by Hitchcock-DeGregori et al. (1985). Recombinant chicken and rat striated $\alpha\alpha$-tropomyosin were expressed in *E. coli* as described by Hitchcock-DeGregori and Heald (1987).

Deconvolution of CD spectra

CD spectra were deconvoluted into component curves by the convex constraint analysis program of Perczel et al. (1991, 1992) as we have described previously (Greenfield & Hitchcock-DeGregori, 1993). The program was a gift of Dr. Gerald D. Fasman.

Molecular modeling

The structure of the AcTM32ox peptide was modeled on that of the GCN4 leucine zipper (O'Shea et al., 1991). The coordinates of the GCN4 peptide were from the Brookhaven Protein Data Bank, file 2ZTA. The N-terminal amino acid of the GCN4 peptide was deleted and the side chains of the GCN4 peptide, starting at Met-2 (an *a* position), were replaced with the side chains of the first 30 residues of rabbit striated muscle α-tropomyosin (Stone & Smillie, 1978) plus GC. The C-terminal cysteines were joined by a disulfide bond and acetyl groups were added to the N-termini of the 2 chains. The acidic and basic side chains were all assumed to be charged at neutral pH. The dielectric constant was set at 1.0. A structure with minimum energy was computed using the computer program Sybyl (Tripos Associates). The force fields were calculated as described by Clark et al. (1989). The energy minimization was performed using the Powell method (Powell, 1977).

Note added in proof

In confirmation of our results and structural hypothesis, Dr. Peter Kim and his colleagues have found that N-terminal acetylation and an Arg in a *g* position can stabilize a truncated form of the GCN4 leucine zipper (Lumb KJ, Carr CM, Kim PS, submitted to *Biochemistry*).

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References

Ansevin AT, Roark DE, Yphantis DW. 1970. Improved ultracentrifuge cells for high-speed sedimentation equilibrium studies with interference optics. *Anal Biochem 34*:237–261.

Brahms S, Brahms J. 1980. Determination of protein secondary structure in solution by vacuum ultraviolet circular dichroism. *J Mol Biol 138*: 538–545.

Brenner SL, Zlotnick A, Stafford WF. 1990. Rec A protein self-assembly. II. Analytical equilibrium ultracentrifugation studies of the entropy-driven self-association of Rec A. *J Mol Biol 216*:949–964.

Cho YJ, Liu J, Hitchcock-DeGregori SE. 1990. The amino terminus of tropomyosin is a major determinant for function. *J Biol Chem 265*:538–545.

Clark M, Cramer RD III, Opdenbosch NV. 1989. Validation of the general purpose Tripos 5.2 force field. *J Comp Chem 10*:982–1012.

Cohen C, Parry DAD. 1986. Alpha helical coiled coils: A widespread motif in proteins. *Trends Biochem Sci 11*:245–248.

Cohen C, Parry DAD. 1990. α-Helical coiled bundles: How to design an α-helical protein. *Proteins Struct Funct Genet 7*:1–15.

Cooper TM, Woody RW. 1990. The effect of conformation on the CD of interacting helices: A theoretical study of tropomyosin. *Biopolymers 30*:657–676.

Crick FHC. 1952. Is α-keratin a coiled coil? *Nature 170*:882–883.

Engel M, Williams RW, Erickson BW. 1991. Designed coiled-coil proteins: Synthesis and spectroscopy of two 78-residue α-helical dimers. *Biochemistry 30*:3161–3169.

Fairman R, Shoemaker KR, York EJ, Stewart JM, Baldwin RL. 1989. Further studies of the helix dipole model: Effects of a free α-NH_3^+ or α-COO^- group on helix stability. *Proteins Struct Funct Genet 5*:1–7.

Goa J. 1954. A micro biuret method for protein determination. Determination of total protein in cerebrospinal fluid. *Scand J Clin Lab Invest 5*:218–222.

Greenfield NJ, Fasman GD. 1969. Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry 8*:4108–4116.

Greenfield NJ, Hitchcock-DeGregori SE. 1993. Conformational intermediates in the folding of a coiled-coil model peptide of the N-terminus of tropomyosin and $\alpha\alpha$-tropomyosin. *Protein Sci 2*:1263–1273.

Heald RW, Hitchcock-DeGregori SE. 1988. The structure of the amino terminus of tropomyosin is critical for binding to actin in the absence and presence of troponin. *J Biol Chem 263*:5254–5259.

Hitchcock-DeGregori SE, Heald RW. 1987. Altered actin and troponin binding of amino-terminal variants of chicken striated muscle α-tropomyosin expressed in *Escherichia coli*. *J Biol Chem 262*:9730–9735.

Hitchcock-DeGregori SE, Lewis SF, Chou TMT. 1985. Tropomyosin lysine reactivities and relationship to coiled-coil structure. *Biochemistry 24*:3305–3314.

Hodges RS, Saund AK, Chong PCS, St. Pierre SA, Reid RE. 1981. Synthetic model for two-stranded α-helical coiled-coils. Design, synthesis and characterization of an 86-residue analog of tropomyosin. *J Biol Chem 256*:1214–1224.

Hodges RS, Sodek J, Smillie LB, Jurasek L. 1972. Tropomyosin: Amino acid sequence and coiled-coil structure. *Cold Spring Harbor Symp Quant Biol 37*:299–310.

Hodges RS, Zhou NE, Kay CM, Semchuk PD. 1990. Synthetic model proteins: Contribution of hydrophobic residues and disulfide bonds to protein stability. *Peptide Res 3*:123–137.

Hol WGJ. 1985. The effects of the α-helix dipole upon the functioning and structure of proteins and peptides. *Adv Biophys 19*:133–165.

Hol WGJ, van Duijnen PT, Berendsen HJC. 1978. The α-helix dipole and the properties of proteins. *Nature 273*:443–446.

Holtzer ME, Holtzer A. 1992. Uncooperative block in the tropomyosin coiled coil. *J Am Chem Soc 113*:7444–7445.

Hu JC, O'Shea EK, Kim PS, Sauer RT. 1990. Sequence requirements for coiled-coils, analysis with λ repressor-GCN4 leucine zipper fusions. *Science 250*:1400–1403.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*:680–685.

Lau SYM, Taneja AK, Hodges RS. 1984. Synthesis of a model protein of defined secondary and quaternary structure. Effect of chain length on the stabilization and formation of two-stranded α-helical coiled-coils. *J Biol Chem 259*:13253–13261.

Lees-Miller JP, Helfman DM. 1991. The molecular basis for tropomyosin isoform diversity. *Bioassays 13*:429–437.

Lehrer SS. 1978. Effect of an interchain disulfide bond on tropomyosin structure, intrinsic fluorescence and circular dichroism studies. *J Mol Biol 118*:209–226.

Liu S, Stafford WF. 1992. A real-time video-based Rayleigh optical system for an analytical ultracentrifuge allowing imaging of the entire centrifuge cell. *FASEB J 6*:A476.

Marqusee S, Baldwin RL. 1987. Helix stabilization by $Glu^{-}\cdots Lys^{+}$ salt bridges in short peptides of *de novo* design. *Proc Natl Acad Sci USA 84*:8898–8902.

Mattice WL, Tilstra L. 1987. Basis for large differences in the cooperativity of the formation of antiparallel β-sheets and clusters of interacting α-helices in isolated chains. *Biopolymers 26*:203–211.

Nelson JW, Kallenbach NR. 1989. Persistence of the α-helix stop signal in the S peptide in trifluoroethanol solutions. *Biochemistry 28*:5256–5261.

O'Neil KT, Hoess RH, DeGrado WF. 1990. Design of DNA-binding peptides based on the leucine zipper motif. *Science 249*:774–778.

O'Shea EK, Klemm JD, Kim PS, Alber T. 1991. X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled coil. *Science 254*:539–544.

O'Shea EK, Rutkowski R, Kim PS. 1989a. Evidence that the leucine zipper is a coiled coil. *Science 243*:538–542.

O'Shea EK, Rutkowski R, Stafford WF III, Kim PS. 1989b. Preferential heterodimer formation by isolated leucine zippers from Fos and Jun. *Science 245*:646–648.

Ozeki S, Kato T, Holtzer ME, Holtzer A. 1991. The kinetics of chain exchange in two-chain coiled coils: $\alpha\alpha$- and $\beta\beta$-tropomyosin. *Biopolymers 31*:957–966.

Perczel A, Hollósi M, Tusnády G, Fasman GD. 1991. Convex constraint analysis: A natural deconvolution of circular dichroism curves of proteins. *Protein Eng 4*:669–679.

Perczel A, Park K, Fasman GD. 1992. Analysis of the circular dichroism spectrum of proteins using the convex constraint algorithm. A practical guide. *Anal Biochem 203*:83–93.

Phillips GN Jr, Fillers JP, Cohen C. 1986. Tropomyosin crystal structure and muscle regulation. *J Mol Biol 192*:111–131.

Powell MJD. 1977. Restart procedure for the conjugate gradient method. *Math Programming 12*:241–254.

Quiocho FA, Sack JS, Vyas NK. 1987. Stabilization of charges on isolated ionic groups sequestered in proteins by polarized peptide units. *Nature 329*:561–564.

Scholtz JM, Baldwin RL. 1992. The mechanism of α-helix formation by peptides. *Annu Rev Biophys Biomol Struct 21*:95–118.

Scholtz JM, Qian H, York EJ, Stewart JM, Baldwin RL. 1991. Parameters of helix-coil transition theory for alanine based peptides of varying chain length in water. *Biopolymers 31*:1463–1470.

Shoemaker KR, Fairman R, Kim PS, York EJ, Stewart JM, Baldwin RL. 1987a. The C-peptide helix from ribonuclease A considered as an autonomous folding unit. *Cold Spring Harbor Symp Quant Biol 52*:391–398.

Shoemaker KR, Kim PS, Brems DM, Marqusee S, York EJ, Chaiken IM, Stewart JM, Baldwin RL. 1985. Nature of the charged-group effect on the stability of the C-peptide helix. *Proc Natl Acad Sci USA 82*:2349–2353.

Shoemaker KR, Kim PS, York EJ, Stewart JM, Baldwin RL. 1987b. Tests of the helix dipole model for stabilization of α-helices. *Nature 326*: 536–567.

Stone D, Smillie LB. 1978. The amino acid sequence of rabbit skeletal α-tropomyosin. The NH_2-terminal half and complete sequence. *J Biol Chem 253*:1137–1148.

Thompson KS, Vinson CR, Freire E. 1993. Thermodynamic characterization of the structural stability of the coiled-coil region of the bZIP transcription factor GCN4. *Biochemistry 32*:5491–5495.

Wada A. 1976. The α-helix as an electric macro-dipole. *Adv Biophys 9*:1–63.

Whitby FS, Kent H, Stewart F, Stewart M, Xie X, Hatch V, Cohen C, Phillips GN Jr. 1992. Structure of tropomyosin at 9 Ångstroms resolution. *J Mol Biol 227*:441–452.

Woody RW. 1992. Circular dichroism and conformation of unordered polypeptides. *Adv Biophys Chem 2*:37–79.

Zhou NE, Kay CM, Hodges RS. 1992a. Synthetic model proteins: The relative contribution of leucine residues at the nonequivalent positions of the 3–4 hydrophobic repeat to the stability of the two-stranded α-helical coiled-coil. *Biochemistry 31*:5739–5746.

Zhou NE, Kay CM, Hodges RS. 1993. Disulfide bond contribution to protein stability. Positional effects of substitution in the hydrophobic core of the two-stranded α-helical coiled coil. *Biochemistry 32*:3178–3187.

Zhou NE, Zhu BY, Kay CM, Hodges RS. 1992b. The two-stranded α-helical coiled-coil is an ideal model for studying protein stability and subunit interactions. *Biopolymers 32*:419–426.