Conformational intermediates in the folding of a coiled-coil model peptide of the N-terminus of tropomyosin and $\alpha\alpha$ -tropomyosin

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Abstract

Circular dichroism was used to study the folding of $\alpha\alpha$ -tropomyosin and AcTM43, a 43-residue peptide designed to serve as a model for the N-terminal domain of tropomyosin. The sequence of the peptide is AcMDAIKKKMQMLKLDVENLLDRLEQLEADLKALEDRYKQLEGGC. The peptide appeared to form a coiled coil at low temperatures ($<25^\circ\text{C}$) in buffers with physiological ionic strength and pH. The folding and unfolding of the peptide, however, were noncooperative. When CD spectra were examined as a function of temperature, the apparent degree of folding differed when the ellipticity was followed at 222, 208, and 280 nm. Deconvolution of the spectra suggested that at least three component curves contributed to the CD in the far UV. One component curve was similar to the CD spectrum of the coiled-coil α -helix of native $\alpha\alpha$ -tropomyosin. The second curve resembled the spectrum of single-stranded short α -helical segments found in globular proteins. The third was similar to that of polypeptides in the random coil conformation. These results suggested that as the peptide folded, the α -helical content increased before most of the coiled coil was formed.

When the CD spectrum of striated muscle $\alpha\alpha$ -tropomyosin was examined as a function of temperature, the unfolding was also not totally cooperative. As the temperature was raised from 0 to 25°C , there was a decrease in the coiled coil and an increase in the conventional α -helix type spectrum without formation of random coil. The major transition, occurring at 40°C , was a cooperative transition characterized by the loss of all of the remaining coiled coil and a concomitant increase in random coil.

Keywords: circular dichroism; coiled coil; folding; peptide; tropomyosin

The coiled coil is a widespread structural motif (Cohen & Parry, 1986, 1990). It is best known in several fibrous proteins including tropomyosin, myosin, and keratin and is the leucine zipper in a class of DNA-binding proteins (Landschulz et al., 1988; O'Shea et al., 1989). Coiled coils are characterized by the presence of a heptapeptide repeat of hydrophobic amino acids found every first and fourth residue, which forms the interface between the two α -helical chains of the coiled coil. The structure of the coiled

coil was predicted by Crick (1952) and was recently solved by O'Shea et al. (1991).

Short coiled-coil peptides have served as useful models for studying folding of both the α -helix and the coiled coil. Zhou et al. (1992a,c) have suggested that coiled-coil peptides are excellent systems for the study of protein folding using circular dichroism because they contain only one kind of regular secondary structure, the α -helix. Hodges and his colleagues pioneered investigations of the requirements for coiled-coil formation (Hodges et al., 1972, 1981, 1990; Talbot & Hodges, 1982; Lau et al., 1984). They found that the coiled-coil α -helical conformation is stabilized by hydrophobic residues at the first (a) and fourth (d) positions of a repeating heptad (a, b, c, d, e, f, g)_n. Recent studies using synthetic peptides as well as molecular genetic approaches have confirmed this finding and have shown that the helical

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Abbreviations: AcTM43ox, acetylated 43-residue N-terminal tropomyosin-like model peptide (disulfide cross-linked to form a dimer); AcTM43re, reduced acetylated 43-residue N-terminal tropomyosin-like model peptide; DTT, dithiothreitol.

	a1	d1	a2	d2	a3	d3	a4	d4	a5	d5	a6	d6																																	
AcTM43	A	c	M	D	A	I	K	K	K	M	Q	M	L	K	L	D	<u>Y</u>	E	N	<u>L</u>	L	D	R	<u>L</u>	E	Q	<u>L</u>	E	A	D	<u>L</u>	K	A	<u>L</u>	E	D	R	<u>Y</u>	K	Q	L	E	G	G	C
Tropomyosin	A	c	M	D	A	I	K	K	K	M	Q	M	L	K	L	D	K	E	N	A	L	D	R	A	E	Q	A	E	A	D	K	K	A	A	E	D	R	S	K	Q	L	E			
Sequence	1				8				15				22				29				36																								

Fig. 1. The sequence of AcTM43 compared to the first 40 residues of rabbit $\alpha\alpha$ -tropomyosin (Stone & Smillie, 1978). The changed residues are underlined. "a" and "d" refer to the positions in the heptad repeat that form the hydrophobic interface between the two chains in the coiled coil.

propensity of residues in other positions can also contribute to stability (Hu et al., 1990; O'Neil et al., 1990; Zhu et al., 1993).

We became interested in the folding of coiled-coil peptides when we began to use synthetic peptides to investigate the structure and function of the N-terminal region of striated muscle tropomyosin. The N-terminus is crucial for actin binding and regulatory function (Hitchcock-DeGregori & Heald, 1987; Heald & Hitchcock-DeGregori, 1988; Cho et al., 1990). In the work described here, we designed a 43-residue peptide model for the N-terminus of tropomyosin (AcTM43) that would form a coiled coil at room temperature under physiological ionic conditions. Circular dichroism was used to characterize the conformation and stability of the peptide.

In the course of our studies on the AcTM43 peptide, we found that its temperature-dependent folding was not cooperative and could not be described as a two-state process. During folding, a considerable amount of an intermediate with a spectrum similar to that of a conventional α -helix is gained before the formation of the maximal amount of coiled coil. During thermal denaturation of $\alpha\alpha$ -tropomyosin we observed a pretransition, which involved loss of some of the coiled coil and a concomitant gain in the content of an intermediate with a conventional α -helical spectrum. These intermediates were detected because the far-UV spectrum was monitored during folding, as opposed to a single wavelength, e.g., 220 or 222 nm.

Results

Design of an N-terminal tropomyosin peptide

We have used an N-terminal model tropomyosin peptide to investigate the folding of a coiled-coil peptide. The sequence of this peptide is identical to the first 40 residues of rabbit striated $\alpha\alpha$ -tropomyosin, except that most of the a and d positions have been changed to leucine to increase the stability of the coiled coil (Fig. 1). A tyrosine residue, in the a position nearest the C-terminal end, was included as a spectroscopic probe. Gly Gly Cys was added at the C-terminus to allow covalent cross-linking. The N-terminus was acetylated. In addition, one a position was replaced by a valine to increase the variety of the side chains at the coiled-coil interface. The rationale was that some side-

chain diversity might help the peptide fold into a unique structure with minimum thermodynamic energy.

Effects of oxidation and concentration on the thermal stability of the peptide

Circular dichroism measurements suggested that both the oxidized and reduced peptides formed coiled-coil α -helices in buffers with near physiological ionic strength and pH (100 mM NaCl, 20 mM sodium PO₄, pH 7.1). From the ellipticity at 222 nm, both were approximately 80% helical at 2 °C (Gans et al., 1991). The CD spectra were typical of α -helices in a coiled-coil conformation (Cooper & Woody, 1990), with greater ellipticity values at 222 nm than at 208 nm.

Initially we examined the effect of oxidation on the stability of the peptide by measuring the ellipticity at a single wavelength, 222 nm, as a function of temperature (Fig. 2). Interchain disulfide bond formation stabilized the peptide. This result is consistent with other reports that oxidation stabilizes the α -helical conformation in coiled-coil peptides (Lehrer, 1978; O'Shea et al., 1989;

Fig. 2. Effect of oxidation on the folding of AcTM43 (0.07 mg/mL) as a function of temperature in 100 mM NaCl, 20 mM PO₄, pH 7.1, $\pm$ 1 mM dithiothreitol. ○, oxidized; ●, reduced.

Hodges et al., 1990; Engel et al., 1991). The same curves were obtained when the peptide was either heated from 2 to 90 °C or cooled from 90 to 2 °C, suggesting that the data were obtained under conditions of thermodynamic equilibrium.

The ellipticity of the oxidized peptide was independent of peptide concentration. The ellipticity of the reduced peptide displayed a concentration dependence, as might be expected if monomers assemble to form a coiled coil (Fig. 3A). However, the folding, as a function of peptide concentration, was not fully cooperative and was more complex than would be expected from a simple two-state model of a helix-coil transition. The reduced peptide was very stable and retained greater than 50% of its initial negative ellipticity at 70 °C, even at a low concentration, 0.027 mg/mL (Fig. 3B). The ellipticity of the peptide, at this concentration, was almost independent of temperature between 0 and 15 °C. This behavior normally would suggest that the folding of the peptide was nearly complete by 2 °C. However, the spectrum of AcTM43re at 2 °C was not independent of peptide concentration (Fig. 3A), and the curves in Figure 3B did not attain the same asymptotic limiting value. The magnitude of the CD bands increased, and the ratio of the ellipticity at 222 to 208 nm also increased, when the peptide concentration was raised, suggesting that more coiled-coil α -helix was formed.

CD spectra of the TM43 peptides as a function of temperature: Deconvolution into components

The above results suggested that the folding of the TM43 peptide could not be described as a cooperative coiled-coil α -helix to random coil transition. First, the change in ellipticity of the oxidized peptide as a function of temperature was almost linear, i.e., not cooperative, between 20 and 70 °C (Fig. 2). Second, there was the disparity between the stability of the reduced peptide to heat dena-

turation and to dilution (Fig. 3). We decided to examine the entire spectrum of the oxidized and reduced AcTM43 peptide as a function of temperature and concentration to learn if structures other than the coiled-coil α -helix and random coil might contribute to the CD at 222 nm. Others have assumed that only the α -helix and random coil are present during unfolding or folding of coiled coils, without distinguishing between the α -helical and coiled-coil conformations (Lehrer, 1978; O'Shea et al., 1989; Holtzer et al., 1990; Zhou et al., 1992a,c).

The relative degree of folding of the peptide appeared to be different when monitored at different wavelengths. The absolute value (Fig. 4A) and relative values (Fig. 4B) of the ellipticity at 222 nm increased as a function of decreasing temperature and were still increasing between 25 and 0 °C. In contrast, the ellipticity at 208 nm was almost constant between 25 and 0 °C. The reduced 43-mer also differed in folding when monitored at 222 and 208 nm. These differences provided clear evidence that there are more than two components contributing to the CD spectra and suggested that there was a folding intermediate.

To determine the nature of the folding intermediate, we initially used several methods to evaluate which conformations contribute to the spectra in Figure 4A (Greenfield & Fasman, 1969; Brahms & Brahms, 1980; Hennessey & Johnson, 1981; Yang et al., 1986). These methods were unsatisfactory because they do not distinguish between the spectrum of a coiled-coil α -helix (with a rise of 5.15 Å/turn referred to as coiled coil) and the conventional straight α -helix (with a rise of 5.4 Å/turn referred to as α -helix) found in globular proteins (Crick, 1953). In addition, in some of the methods the fractional weights of the evaluated components were negative, and/or the sum of weights was greater than one. On the other hand, the data were very well fit by a nonconstrained linear combination of the spectra of $\alpha\alpha$ -tropomyosin, which is almost pure coiled coil (Phillips et al., 1986); the α -helix derived from

Fig. 3. **A:** Spectrum of AcTM43re at three different peptide concentrations. The buffer is the same as in Figure 2. **B:** Effect of concentration on the change in the mean residue ellipticity at 222 nm of AcTM43re as a function of temperature. $\circ$, 1.0 mg/mL; $\bullet$, 0.17 mg/mL; ∇ , 0.027 mg/mL at 2 °C.

Fig. 4. A: Effect of temperature on the CD spectrum of AcTM43ox, 0.1 mg/mL. ○, 2 °C; ●, 10 °C; ▽, 25 °C; ▼, 50 °C; □, 70 °C; ■, 90 °C. The buffer is the same as in Figure 2. Fourteen data sets were collected, but only six are shown for the sake of clarity. **B:** Relative changes in ellipticity at 222 (○) and 208 (●) nm of the data in A between 0 and 90 °C as a function of temperature. The data shown are from a single experiment, but the differences were reproducible in five independent experiments.

the spectrum of a globular protein, myoglobin (Brahms & Brahms, 1980); and a random coil (Brahms & Brahms, 1980); and the sums of the fractional weights were close to one. At low temperatures (<25 °C) the spectrum was predominantly that of the coiled coil. Between 25 and 70 °C, the apparent amount of coiled-coil spectrum decreased but the α -helix spectrum increased. Above 70 °C, the spectrum of the peptide appeared to be a mixture of the spectra of the α -helix and random coil. The data in Figure 4A were better fit using both forms of helix as standards ($4.5 \pm 1.3\%$ [mean $\pm$ standard deviation] between the calculated curves and experimental data) than when either the α -helix or the coiled-coil helix was used alone ($7.2 \pm 3.0\%$ and $8.2 \pm 12.5\%$, respectively).

We were concerned that our selection of reference spectra might introduce bias into the analysis of the data. As an alternative, we used the convex constraint algorithm of Perczel et al. (1991) to deconvolute the spectra. This algorithm allows deconvolution of sets of spectra into component curves without prior knowledge of the structures that contribute the chiral elements. In this method, the fractional weights of all components must be positive and the sum of the weights is constrained to equal one.

Figure 5A shows the results of deconvoluting the data for AcTM43ox shown in Figure 4A into three curves. The three component spectra resemble (1) native tropomyosin (representative of a coiled-coil conformation), (2) the α -helix (Brahms & Brahms (1980), and (3) a mixture of

Fig. 5. A: The three basis curves that contribute to the spectra in Figure 4A, which were resolved using the convex constraint algorithm of Perczel et al. (1991). **B:** Temperature-dependent change in the relative weights of each curve in A to the spectra of AcTM43ox. ○, coiled coil; ●, α -helix; ▽, mixture of α -helix and random coil.

the α -helical and random coil spectra (Greenfield & Fasman, 1969). Figure 5B illustrates the fractional contribution of each deconvoluted spectrum to the observed CD spectrum as a function of temperature. The temperature-dependent changes in the weights of the component curves were similar to those seen using reference spectra for the coiled coil, α -helix, and random coil. When the 14 spectra were deconvoluted into two curves rather than three, the standard deviation between the calculated data matrix and the experimental raw data matrix increased twofold from 1.0% to 2.0%. Increasing the number of basis curves from three to four, on the other hand, resulted in only a slight decrease in the standard deviation to 0.7%. Moreover, two of the extracted curves were almost identical to each other when the data were deconvoluted into four curves.

A problem with the curves in Figure 5A is that one is a mixture of α -helix and random coil. This is a consequence of not adding reference spectra, since the convex constraint algorithm can only distinguish a chiral component if it represents at least 50% of one of the curves in the data set (Perczel et al., 1992). It was necessary to include reference spectra to totally resolve the curves into components using the convex constraint algorithm.

Figure 6A illustrates the deconvoluted spectra that were output using the convex constraint algorithm when reference curves for four secondary structures (Brahms & Brahms, 1980; see Materials and methods) and $\alpha\alpha$ -tropomyosin were added to the data set in Figure 4A. The curves that were output for the β -pleated sheet, β -turn, and random coil were unchanged compared to the reference data used as input. One resolved curve was

similar, but not identical, to the α -helical reference spectrum. This spectrum also resembles the deconvoluted spectrum for the α -helix that was obtained by Perczel et al. (1991) when they performed convex analysis on CD data from 18 globular proteins over a wavelength range of 240–190 nm. The fifth curve was similar to the spectrum of native $\alpha\alpha$ -tropomyosin. The standard deviations between the calculated curves and the 14 sets of experimental data in Figure 4 averaged $1.3 \pm 0.5\%$. This fit was much better than the fits obtained using basis sets derived from peptides (Brahms & Brahms, 1980) or proteins (Yang et al., 1986; Perczel et al., 1992), which gave standard deviations of $11.8 \pm 5.6\%$, $4.2 \pm 1.3\%$, and $19.1 \pm 12.0\%$, respectively.

During folding, as the temperature was reduced, the percentage of the coiled coil decreased while the α -helical component first increased and then began to decrease (Fig. 6B). The coiled coil was almost totally lost before the random coil appeared. There was almost no contribution of β -turn or β -pleated sheet to the spectrum of the AcTM43ox peptide at any temperature. The deconvolution results suggest that whereas the α -helix of the AcTM43 peptide is very stable, with a T_M of formation $>90^\circ\text{C}$, the coiled coil is much less stable and appreciable coiled coil is only found below 25°C . The results obtained for the oxidized peptide were independent of concentration over a range of 0.03–0.11 mg/mL.

When CD spectra of the reduced peptide were deconvoluted, the component curves were similar to those found with AcTM43ox (data not shown). Oxidation of the peptide had only a small effect on the fraction of the coiled-coil spectrum (Fig. 7A) as a function of temperature, but

Fig. 6. A: Basis spectra derived from deconvoluting the data in Figure 4A into five basis curves using the convex constraint algorithm of Perczel et al. (1991). Reference spectra from the α -helix, β -pleated sheet, β -turn and random coil (Brahms & Brahms, 1980), and $\alpha\alpha$ -tropomyosin were added to the data set before analysis. The output curves are similar to the coiled coil ($\square$), α -helix ($\circ$), random coil (∇), β -structure ($\bullet$), and β -turn ($\blacktriangledown$). **B:** Temperature-dependent change in the weights of the curves in A that contribute to the spectra of AcTM43ox (symbols as in A).

Fig. 7. Effect of oxidation on the weights of the curves that contribute to the spectra of the peptide, 0.1 mg/mL, as a function of temperature. **A:** Coiled coil. **B:** α -Helix. **C:** Random coil. $\circ$, AcTM43re; $\bullet$, AcTM43ox.

stabilized the amount of the intermediate α -helical spectrum (Fig. 7B).

CD of the tyrosine residue of AcTM43re as a function of temperature

In our design of AcTM43 we placed a Tyr in the penultimate C-terminal α position to serve as a spectroscopic probe. Cooper and Woody (1990) have calculated that tyrosine in the α position has an enhanced ellipticity compared to tyrosine in an α -helix or random coil. The midpoint for the ellipticity change at 280 nm was approximately 25 °C (Fig. 8A). Analysis of the far-UV spectra in Figure 8B showed that at the high peptide concentration used, 6 mg/mL, only two conformations contribute, the coiled coil and α -helix. Even at 70 °C the peptide appeared to be completely helical, although the ellipticity

arising from the tyrosine residue was almost completely lost. The loss of tyrosine CD has a temperature dependence similar to that of the loss of the coiled-coil spectrum. A similar result was obtained when the aromatic spectrum of AcTM43ox, 0.07 mg/mL, was examined. The differences between the temperature-dependent change of tyrosine ellipticity and the change of total α -helical content are further evidence that the folding of the AcTM43 peptide is not cooperative.

CD spectrum of the AcTM43 peptide in 50% trifluoroethanol as a function of temperature

We have considered the possibility that the intermediate α -helical spectrum observed during the folding of the AcTM43 peptide might be an artifact of the deconvolution method. Hodges et al. (1990) have suggested that peptides lacking a disulfide bond, with a repeating hep-

Fig. 8. **A:** Change in the molar ellipticity of AcTM43re, 6 mg/mL, as a function of temperature at 280 nm. The buffer is the same as in Figure 2. **B:** The mean residue ellipticity in the far UV of AcTM43re, 6 mg/mL at different temperatures. $\circ$, 5 °C; $\bullet$, 15 °C; ∇ , 25 °C; $\blacktriangledown$, 50 °C; $\square$, 70 °C.

tad motif, do not form coiled coils in 50% trifluoroethanol, although they are highly helical. In the presence of 50% trifluoroethanol there was no evidence for a folding intermediate. In contrast to the results in buffer alone (Fig. 4A), the CD spectra of AcTM43re displayed a clear isosbestic point, and the relative changes in ellipticity at 222 and 208 nm as a function of temperature were identical (Fig. 9A). When the spectra were deconvoluted (Fig. 9B), two of the curves (open circles and filled circles) were almost identical to each other, suggesting that only two components are necessary to fully describe the spectral data set. Neither curve resembles the coiled-coil spectrum. The weighted fractions of the two almost identical curves oscillated randomly as a function of temperature (Fig. 9C). Results obtained with the oxidized peptide (data not shown) were similar to those obtained with the reduced peptide. Furthermore, another tropomyosin peptide that folds cooperatively does not exhibit a spectral intermediate (Greenfield, unpubl.).

Deconvolution of spectra of AcTM43re obtained as a function of concentration

We were concerned that the observed spectrum of the helical intermediate might be the result of simple linear temperature-dependent changes in the CD spectrum of the coiled coil rather than an effect on peptide conformation. This appeared unlikely, because the slope of the ellipticity change at 222 nm of the AcTM43ox peptide, between 20 and 70 °C, was almost twice the slope of the change in ellipticity between 0 and 10 °C (Fig. 2). To further test this possibility, we deconvoluted spectra of AcTM43re obtained as a function of concentration at 10 °C. As the samples were diluted, the ratio of the ellipticity at 222 to 208 nm decreased. The changes were similar to those seen for the peptide upon dilution at 2 °C (Fig. 3A). Deconvolution of the spectra showed the same

conformational components that were obtained as a function of temperature (see Fig. 6). Upon dilution, the percentage of the coiled-coil spectrum decreased and the percentage of the α -helical and random coil spectra both increased. The contribution of the random coil component in any of the curves obtained over a concentration range of 0.2 to 1,200 μ M peptide at 10 °C was low, <20%. These results confirm the presence of a folding intermediate and argue that it is not simply an effect of temperature on the spectrum of the coiled coil.

Deconvolution of the CD spectra of $\alpha\alpha$ -tropomyosin obtained as a function of temperature

We examined the temperature dependence of the CD spectrum of $\alpha\alpha$ -tropomyosin to learn if there were folding intermediates in this well-characterized coiled-coil protein. As in the case of the TM43 peptide, the fractional changes of the ellipticity of reduced $\alpha\alpha$ -tropomyosin at 222 nm and 208 nm, as a function of temperature, were not identical (Fig. 10A). Below 20 °C the ellipticity at 222 nm changed more than that at 208 nm (Fig. 10B). The spectra in Figure 10A were deconvoluted into five curves that were very similar to those obtained with the TM43 peptide (Fig. 6A). The change in the percentage of the coiled-coil spectrum as a function of temperature showed at least two transitions (Fig. 10C), consistent with previous CD measurements (Woods, 1969; Lehrer, 1978; Holtzer et al., 1983; Mo et al., 1990). The transition at approximately 20 °C was gradual, and was accompanied by a gain in the α -helical spectrum but no gain in the random coil. The second transition, near 40 °C, was steep and cooperative; the loss of coiled coil was accompanied by an increase in random coil. The results obtained with oxidized $\alpha\alpha$ -tropomyosin were similar (data not shown). The spectrum of the helical intermediate was also ob-

Fig. 9. **A:** The CD of AcTM43re as a function of temperature in 50% trifluoroethanol, 100 mM NaCl, 20 mM PO₄, pH 7.1. $\circ$, 2 °C; $\bullet$, 10 °C; ∇ , 25 °C; $\blacktriangledown$, 50 °C; $\square$, 70 °C. **B:** Three basis curves derived from the spectra in A using the convex constraint algorithm of Perczel et al. (1991). $\circ$, α -helix; $\bullet$, α -helix; ∇ , α -helix and random coil. **C:** The change in the weights of each curve in B, which contribute to the spectra of AcTM43re, 0.05 mg/mL, in 50% trifluoroethanol as a function of temperature.

Fig. 10. **A:** CD spectra of reduced chicken striated muscle $\alpha\alpha$ -tropomyosin, 0.15 mg/mL, as a function of temperature between 2 and 70 °C. The buffer is the same as in Figure 2. **B:** Relative temperature-dependent change of ellipticity at 222 (○) and 208 (●) nm of reduced $\alpha\alpha$ -tropomyosin. **C:** The change in the fraction of each deconvoluted curve contributing to the spectra of reduced $\alpha\alpha$ -tropomyosin as a function of temperature. □, coiled coil; ○, α -helix; ▽, random coil; ●, β -pleated sheet; ▼, β -turn.

served with oxidized and reduced tropomyosin in non-polymerizing conditions in 600 mM NaCl, 20 mM sodium phosphate, pH 7.1.

Discussion

The results presented here show that the folding of both the oxidized and reduced AcTM43 peptide cannot be described by a single cooperative transition between an unfolded and a coiled-coil state, and suggest the presence of an α -helical folding intermediate. First, the relative change of ellipticity as a function of temperature is different when followed at different wavelengths. Second, the ellipticity of AcTM43ox at 222 nm as a function of temperature is almost linear, i.e., not cooperative. Third, deconvolution of the CD spectra gives evidence for a folding intermediate that has a high content of α -helix but little or no coiled coil.

We did not anticipate this result based on most previous work on coiled-coil peptides. Other investigators have described the folding of many coiled-coil peptides as cooperative, two-state helix-coil transitions (O'Shea et al., 1989; Holtzer et al., 1990; Zhou et al., 1992a,c). In these analyses the α -helix was detected using CD at only one wavelength (220 or 222 nm), and therefore spectral changes such as we have reported here went unnoticed. Engel et al. (1991), however, did report the presence of an intermediate during thermal denaturation of a 39-residue coiled-coil peptide. Their far-UV spectra showed changes similar to those we have reported here, but they were not analyzed for the contribution of different helical structures. Holtzer and Holtzer (1991) described the folding of a 35-residue tropomyosin peptide (residues 16–46). This peptide was partially helical, but the folding showed no concentration dependence. They suggested this peptide

represents a noncooperative region, but they did not analyze the spectra for different secondary structures.

We will now consider what the intermediate helical conformation may be in terms of the overall secondary structure and the relationship between the intermediate and assembly of the coiled coil. We realize that circular dichroism alone cannot distinguish between the possible models. The most simplistic interpretation is that the α -helical conformation is comparable to that seen in globular proteins and that the chains form an α -helical monomer before they dimerize to form a coiled coil. Arguing against this model is that, with few exceptions, exposed α -helices are not found in protein structures, and α -helical peptides are generally unstable in aqueous solution. (For a review, see Scholtz & Baldwin [1992].) It is also inconsistent with equilibrium sedimentation analysis of the peptide (see below).

Another possible model is that the α -helix develops in partially associated peptides before the development of the fully aligned coiled coil. The α -helices in the intermediate may be stabilized by interpeptide hydrophobic interactions similar to those seen between amphipathic helices in globular proteins. Disulfide bond formation stabilizes the α -helix, presumably by increasing the likelihood of both intra- and interchain hydrophobic interactions (Kim & Baldwin, 1990). This model accommodates a folding intermediate, which is part coiled coil and part α -helix, for example, a coiled coil with helical frayed ends, as suggested by the tyrosine CD analysis. This model is consistent with the results of Zhou et al. (1992b), who have found that leucine residues at the ends of a coiled-coil model peptide are less buried than those in the middle.

We have described the folding intermediate as being α -helical based on the similarity of its spectrum to that of short α -helices found in globular proteins. However, we realize that the spectrum may arise from other confor-

mations, including helices in a "molten globule" state, or the 3_{10} helix. Manning and Woody (1991) have calculated that the spectrum of a 3_{10} helix is similar to that of an α -helix. Although there is controversy concerning the definition of the molten globule, it has been described as having a high content of secondary structure, which may or may not be in dynamic equilibrium with less ordered structures (Ptitsyn, 1987; Kuwajima, 1989; Friere & Murphy, 1991; Murphy et al., 1992). Raleigh and DeGrado (1992) have described the thermal transitions of a designed peptide with a helix-loop-helix motif. NMR experiments suggest that this peptide is fully folded below room temperature but forms a molten globule above 25 °C. CD experiments showed the loss of aromatic bands over a temperature range (0–35 °C) where the ellipticity at 222 nm changes only slightly, similar to what we have observed with the AcTM43 peptide.

We originally interpreted our results with the assumption that the AcTM43 peptide would form a conventional two-chain coiled coil. However, recent analysis of both the oxidized and reduced peptides using sedimentation equilibrium centrifugation has shown that they assemble to form tetramers (Walter Stafford, pers. comm.). The K_A 's of the reduced peptide for the dimer to tetramer equilibrium were almost independent of temperature, 46 L/g at 4 °C and 30 L/g at 30 °C. There was almost no monomer present at either temperature. It is unlikely that the two types of helical spectra we observed are a consequence of the dimer to tetramer equilibrium. First, the spectral changes do not correlate in a simple fashion with the changes in association. For example, at a peptide concentration of 6 mg/mL, the contribution of the helical folding intermediate to the spectrum of AcTM32re increased from 30 to 50% between 5 and 25 °C. At this concentration, the peptide is almost 100% tetrameric at both temperatures. Second, the CD results obtained with the oxidized peptide were independent of peptide concentration, indicating that the spectral change is not due to the dimer-tetramer equilibrium. Third, the spectral intermediate was also observed during the folding of chicken muscle $\alpha\alpha$ -tropomyosin under conditions where it does not polymerize, i.e., in buffers containing high salt (0.6 M NaCl).

We have also analyzed the folding of a well-characterized coiled-coil protein, chicken striated muscle $\alpha\alpha$ -tropomyosin. The temperature-dependent changes in the CD spectrum of tropomyosin suggest that the pretransition may involve formation of a partially unfolded structure (possibly a molten globule) with a high helical content but little or no random coil, similar to the folding intermediate that we see in the AcTM43 peptide. Lehrer and coworkers have previously suggested that the pretransition of tropomyosin may represent a partially denatured state in which the unfolded regions of the chains move more closely together (Graceffa & Lehrer, 1980; Betteridge & Lehrer, 1983; Ishii et al., 1992). Lehrer has suggested that the denatured segments may be in a molten globule state (S.S. Lehrer, pers. comm.).

This interpretation differs from those previously proposed. Holtzer and Holtzer (1992) suggested that the pretransition of tropomyosin involves fraying at the ends of the molecule with an increase in random coil. Hvidt et al. (1985) observed a linear change in the optical rotatory dispersion of α -tropomyosin at 233 nm below 25 °C, comparable to the change we observe in the CD of tropomyosin at 222 nm. There was no concomitant change in the b_0 parameters of the Moffitt equation, similar to the lack of observed change in the CD at 208 nm. They suggested that the observed pretransition of tropomyosin may result from general changes in the optical properties of all α -helices as a function of temperature. However, Holtzer and Holtzer (1992) found that the slope of the change of ellipticity with temperature in the pretransition region varies greatly for different coiled-coil proteins, showing that there is not a simple single temperature effect on the spectrum of the coiled coil. Our results with the AcTM43 peptide also suggest that the pretransitions of tropomyosins are not due to temperature effects on the CD spectrum per se of the α -helix. First, the spectrum of the folding intermediate is observed when the peptide is diluted at 10 °C. Second, the relative ratios of the coiled-coil spectrum and the " α -helix" spectrum of the reduced and oxidized peptide and $\alpha\alpha$ -tropomyosin, as a function of temperature, are quite distinct from one another. Finally, no folding intermediate is seen when the peptide is examined in 50% trifluoroethanol.

Our study of the folding of the tropomyosin peptide clearly shows that analysis of the temperature-dependent change of the entire CD spectrum gives more information about the folding of coiled coils than measuring the change in ellipticity at a single wavelength. In assessing factors that affect the stability of α -helices and coiled coils, it is important to realize that they are different structures and that their conformational transitions are not necessarily linked. The results also emphasize the utility of the convex constraint algorithm of Perczel et al. (1991) for evaluating the contribution of different secondary structures to CD spectra.

Materials and methods

Peptide synthesis, purification, and characterization

The peptide was synthesized and purified by the Protein Chemistry Laboratory at the University of Pennsylvania on an automated Milligen 9050 Solid Phase Peptide Synthesizer using 9-fluorenyl methoxy carbonyl amino acids with either pentafluorenyl ester or 1-oxo-2-hydrobenoxotriazine active groups and protected side chains. The C-terminal amino acid was coupled to polyamide Kieselguhr resin (Pepsyn K resin). Deprotection was performed in 20% piperidine. The coupling reaction was performed in 0.33 M 1-hydroxybenzotriazole and washed in dimethylformamide. Following synthesis, the peptide was cleaved by treatment with 90% trifluoroacetic acid in the

presence of 5% thioanisole, 3% 1,2-ethanedithiol, and 2% anisole. The cleaved peptide was then purified by reverse-phase high performance liquid chromatography on a C₁₈ column. The peptide was synthesized with the N-terminal Met acetylated and unacetylated. The identity of the purified peptide was confirmed by sequencing of the unacetylated peptide on an automated Milligen 6600 Solid Phase Protein/Peptide Sequencer. The mass of the acetylated peptide was confirmed by ESI mass spectroscopy at the CATF Mass Spectrometry Support Facility at Rutgers University.

Striated $\alpha\alpha$ -tropomyosin was isolated and purified from chicken pectoral muscle as described by Hitchcock-DeGregori et al. (1985).

Preparation of fully oxidized and reduced peptides

The AcTM43 peptide was fully reduced when it was incubated with 1 mM DTT for 10 min at 70–90 °C. AcTM43 was oxidized by incubating solutions, 10–20 mg/mL, in 100 mM NaCl, 20 mM sodium PO₄, pH 7.1 (buffer 1), with a 3-fold excess of K₃Fe(CN)₆ and 0.3-fold addition of catalytic CuSO₄ as described by Ozeki et al. (1991). The reaction mixtures were incubated at room temperature overnight, and the reactions were stopped by adding a 10-fold excess of potassium EDTA, pH 8.0. The oxidized peptide was separated from the other components of the reaction mixture by chromatography on a Shodex WS-8025 size-exclusion column equilibrated with buffer 1. The isolated reduced and oxidized peptides migrated as separate bands when analyzed by sodium dodecyl sulfate gel electrophoresis in the absence of reducing agents (Laemmli, 1970). When the oxidized peptide was boiled with DTT, its migration was identical to the reduced peptide. The concentrations of peptide stock solutions (0.1–6.0 mg/mL) were estimated by the micro-biuret procedure of Goa (1954), using bovine serum albumin as a standard.

Circular dichroism measurements

CD measurements were performed on an Aviv model 62 DS spectropolarimeter fitted with a thermal regulated cell holder in 0.1–1.0-cm rectangular cuvettes. Additional measurements were made in water-jacketed cylindrical cuvettes (0.01 and 1.0 cm). The temperature was controlled using a Haake model CH constant temperature bath fitted with a Haake model F3 temperature controller.

The temperature dependent folding and unfolding were followed in two ways. When the ellipticity was monitored at 222 nm, the temperature of the cuvette was either lowered or raised in 2 °C increments from 2 to 90 °C at 1-min intervals. In experiments monitoring the entire spectrum, the temperature was raised in 5–10 °C increments with 5-min equilibration.

The helical contents of the peptides were estimated using the equation for the chain length dependence of an

α -helix, $[\theta]_{222} = 40 \times 10^3 \times (1 - 4.6/N) = 36 \times 10^3$ deg·cm²/dmol (Gans et al., 1991).

Deconvolution of CD spectra

At first, data expressed in deg·cm²/dmol were fit to the reference spectra of a coiled coil ($\alpha\alpha$ -tropomyosin obtained at 2 °C in 2.5 M NaCl, 20 mM PO₄, pH 7.1), the α -helix, and random coil (Brahms & Brahms, 1980) using a linear nonconstrained method of least-squares procedure. A program supplied by Aviv Associates (Lakewood, New Jersey) was used to perform the calculations. Subsequently, the convex constraint analysis program of Perczel et al. (1991) was used to deconvolute the spectra. Data were deconvoluted both with and without the addition of five reference spectra. The reference spectra were the α -helix, β -turn, β -pleated sheet, and random coil of Brahms and Brahms (1980), and tropomyosin in high salt, described above.

Evaluation of the goodness of fit

The standard deviations between the theoretical curves obtained using the various deconvolution methods and the experimental data were evaluated using the Lincomb program of Perczel et al. (1992). This method assumes that the measured CD spectrum, $S(\lambda)$, at each temperature is a weighted sum of pure component reference spectra, $\Theta_i(\lambda)$, with weights, w_i ; that w_i is not negative; and that the sum of the weights equals one.

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