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Unfolding domains of recombinant fusion αα-tropomyosin

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Abstract

The thermal unfolding of the coiled-coil α-helix of recombinant αα-tropomyosin from rat striated muscle containing an additional 80-residue peptide of influenza virus NS1 protein at the N-terminus (fusion-tropomyosin) was studied with circular dichroism and fluorescence techniques. Fusion-tropomyosin unfolded in four cooperative transitions: (1) a pretransition starting at 35 °C involving the middle of the molecule; (2) a major transition at 46 °C involving no more than 36% of the helix from the C-terminus; (3) a major transition at 56 °C involving about 46% of the helix from the N-terminus; and (4) a transition from the nonhelical fusion domain at about 70 °C. Rabbit skeletal muscle tropomyosin, which lacks the fusion peptide but has the same tropomyosin sequence, does not exhibit the 56 °C or 70 °C transition. The very stable fusion unfolding domain of fusion-tropomyosin, which appears in electron micrographs as a globular structural domain at one end of the tropomyosin rod, acts as a cross-link to stabilize the adjacent N-terminal domain. The least stable middle of the molecule, when unfolded, acts as a boundary to allow the independent unfolding of the C-terminal domain at 46 °C from the stabilized N-terminal unfolding domain at 56 °C. Thus, strong localized interchain interactions in coiled-coil molecules can increase the stability of neighboring domains.

Keywords: circular dichroism; coiled-coil; domains; electron microscopy; pyrene excimer; recombinant; tropomyosin; tryptophan fluorescence

It has been proposed that amino acid sequences encoded by exons may represent structural domains involved in function (Gilbert, 1985). Although striated muscle tropomyosin is constructed from an mRNA derived from nine coding exons (Ruiz-Opazo et al., 1985), structural domains are not apparent in the 400-Å α-helical coiled-coil molecule, which is continuous and uninterrupted except possibly for several residues at the ends (Phillips et al., 1986). Yet there are specific interactions with other proteins of the muscle thin filament, whereby one tropomyosin molecule interacts with seven contiguous actin subunits and one troponin complex (Leavis & Gergely, 1984; Zot & Potter, 1987). Differential calorimetric studies of striated αα-tropomyosin have been interpreted as having seven unfolding domains (Potekhin & Privalov, 1982), suggesting a relationship to actin binding sites. Analyses of the distribution of charged residues along the sequence suggested either two (McLachlan & Stewart, 1976) or one group (Phillips et al., 1986) of seven regions capable of interacting with actin. In contrast to calorimetric data, however, circular dichroism (CD) studies on striated tropomyosin only show one major unfolding transition (Woods, 1969; Lehrer, 1978; Holtzer et al., 1983) with a broad unfolding pretransition involving at least 20% of the α-helix (Woods, 1976; Betteridge & Lehrer, 1983). Fluorescence (Graceffa & Lehrer, 1980; Betteridge & Lehrer, 1983) spin label (Graceffa & Lehrer, 1984), and enzyme digestion studies (Ueno, 1984) suggest that the region that unfolds in the pretransition is approximately bounded by residues 130–190.

In this study on a recombinant rat striated fusion αα-tropomyosin (fusion-tropomyosin), information on the location of regions in tropomyosin that cooperatively unfold was obtained. Fusion-tropomyosin contains an ad-

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ditional peptide at the N-terminus of the tropomyosin sequence consisting of the first 80 residues of a nonstructural protein, NS1, of a type A influenza virus (Baez et al., 1980). The fusion peptides of the two chains interact to form a globular domain seen at one end of the tropomyosin rod in electron micrographs. As in the case of tropomyosin, for fusion-tropomyosin the least stable region appears to be located in the middle of the molecule including Cys 190. However, in contrast to tropomyosin, for which the remaining helix unfolds in a single transition, the C- and N-terminal domains of fusion-tropomyosin unfold in second and third transitions at successively higher temperatures. It appears that the fusion domain, which remains folded during these transitions, acts as an interchain cross-link to stabilize the N-terminal domain to which it is attached and the unfolded middle of the molecule determines the C-terminal boundary of the N-terminal domain.

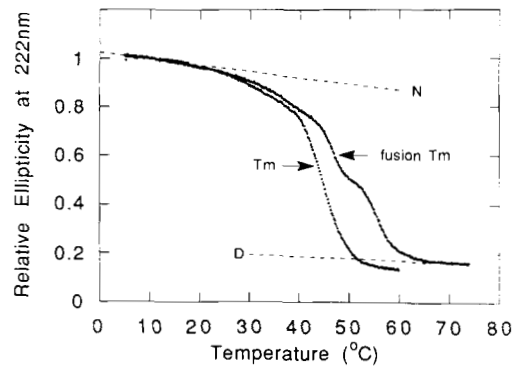

Fig. 1. Comparison of thermal helix unfolding profiles of fusion-tropomyosin (fusion Tm) with tropomyosin (Tm). The ellipticity was normalized at 10 °C. The dashed lines indicate the temperature dependence of the ellipticity in the native (N) and denatured (D) states calculated from the fusion Tm data between 5 and 20 °C and 70 and 75 °C, respectively. [Tm] = [fusion Tm] = 0.04 mg/mL in 0.5 M NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, 10 mM Na-phosphate buffer, pH 7.5.

Results

The helix thermal unfolding profile of fusion-tropomyosin was compared with that of striated muscle $\alpha\alpha$-tropomyosin (Fig. 1). In spite of the presence of the fusion peptide, the molar ellipticity at 222 nm at 10 °C of fusion-tropomyosin was 1.76×10^{11} deg cm^{-1} M^{-1}, similar to that of tropomyosin (1.87×10^{11} deg cm^{-1} M^{-1}). Thus, the fusion peptide attached to tropomyosin appears to contain little or no α-helix. The unfolding profile of fusion-tropomyosin showed a small pretransition between 30 °C and 40 °C and two approximately equal main transitions at 46 °C and 56 °C, in contrast to tropomyosin whose helix unfolds in a single 45 °C main transition. With the assumption of a linear temperature dependence of ellipticity at temperatures below all of the transitions, it is estimated that 18% of the original helix is lost below 41 °C (part of the pretransition), 36% between 41 °C and 51 °C (the remaining pretransition and most of the first main transition), and 46% above 51 °C (the second main transition). These results show that a large region of the tropomyosin helix is stabilized by the fusion peptide, while another large region is unaffected, each behaving as cooperative unfolding domains. The helix thermal unfolding profile of fusion-tropomyosin did not change when the protein concentration was increased from 0.04 mg/mL to 1.0 mg/mL, indicating that the stabilization is not due to protein association.

To determine which part of tropomyosin was stabilized by the fusion peptide, the temperature dependence of the Tyr CD signal at 280 nm (Chao & Holtzer, 1975; Bullard et al., 1976; Lehrer, 1978) was monitored as a probe of the unfolding of the C-terminal domain, because five out of the six Tyr are located in the C-terminal half of tropomyosin (Stone & Smillie, 1978). The CD spectrum of fusion-tropomyosin in the near UV region was very similar to that of tropomyosin, suggesting that the Tyr residues are in a similar environment in the two proteins and that the single Trp of the fusion peptide does not appreciably contribute. The temperature dependence of the Tyr signal of fusion-tropomyosin showed only one transition at 45 °C, corresponding to the first main transition of the helix unfolding profile of fusion-tropomyosin (Fig. 2). Thus, the C-terminal domain most likely unfolds in the first main helix transition and the N-terminal domain of tropomyosin, which unfolds at 56 °C is the domain that is stabilized by the fusion peptide. The Tyr CD data for tropomyosin show one transition a few degrees lower than for fusion-tropomyosin (Fig. 2), indicating that the C-terminal half is also slightly stabilized by the presence of the fusion peptide.

The fusion peptide contains a Trp residue at fusion sequence position 11 (Baez et al., 1980) and there are no

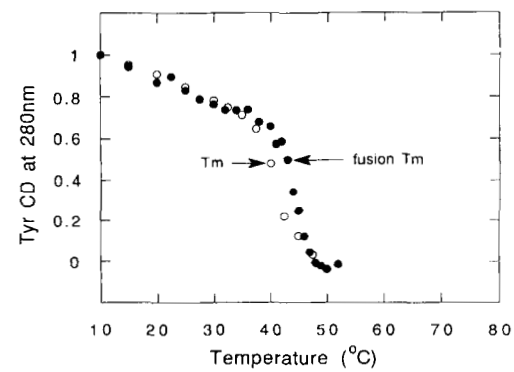

Fig. 2. Temperature dependence of the normalized Tyr CD signal at 280 nm of fusion-tropomyosin and tropomyosin. $\theta_{280\,nm}$ = 224 and 208 deg·cm^2 (mmol of protein)$^{-1}$ at 10 °C for fusion Tm and Tm, respectively. Solvent conditions were the same as in Figure 1.

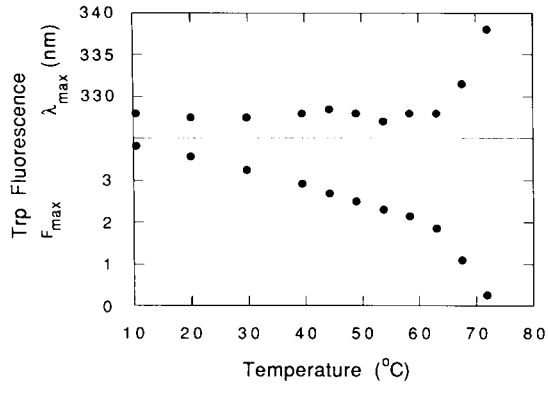

Fig. 3. Temperature dependence of the Trp fluorescence wavelength maximum (top) and intensity (bottom) of fusion-tropomyosin. Excitation wavelength = 295 nm; [fusion-tropomyosin] = 0.1 mg/mL in solvent of Figure 1.

Trps in tropomyosin. The stability of the fusion peptide region of fusion-tropomyosin was therefore studied by monitoring its Trp fluorescence. The Trp residue in the fusion peptide was relatively inaccessible to solvent below 65 °C as indicated by its blue-shifted emission peak wavelength (327 nm) (Fig. 3). Above 65 °C, there was a red shift of the spectrum and a corresponding decrease in the fluorescence intensity, indicating that the Trp became exposed to solvent, due to the unfolding of the fusion peptide. Thus, the fusion peptide region is considerably more stable than the tropomyosin part to which it is attached and unfolds independently from the tropomyosin part as a fusion domain. Between 60 °C and 70 °C, there was no change in the ellipticity at 222 nm of the fusion-tropomyosin confirming that although the fusion domain unfolds to expose its Trp residue, it does not contain much helix, in agreement with the above measurement of molar ellipticity of fusion-tropomyosin.

There are 2 Cys residues in fusion-tropomyosin, one at position 190 in the tropomyosin sequence and one at position 13 in the fusion peptide sequence. The excimer fluorescence of pyrene-labeled Cys was used to probe the thermal stability of the tropomyosin region near Cys 190 (Betcher-Lange & Lehrer, 1978; Graceffa & Lehrer, 1980; Ishii & Lehrer, 1990) and the fusion domain. The pyrene excimer/monomer fluorescence intensity ratio at 10 °C for fusion-tropomyosin (1.44) was greater than tropomyosin (0.31), indicating that there were contributions to the excimer fluorescence from pyrenes in the fusion domain. This was verified with the temperature dependence of the excimer fluorescence intensity (Fig. 4). The fluorescence increase between 30 °C and 40 °C and the decrease between 40 °C and 50 °C most probably originates from Cys 190 because the fluorescence of pyrene at Cys 190 of tropomyosin shows similar behavior (Fig. 4). Because the increase in excimer fluorescence of tropomyosin is due to local unfolding in the pretransition (Graceffa & Lehrer, 1980) the similar increase for fusion-tropomyosin indicates a similar unfolding. The similar decrease in excimer fluorescence for fusion-tropomyosin and tropomyosin above 40 °C indicates further separation of the two tropomyosin chains to include the middle region. The remaining excimer fluorescence contribution above 50 °C for fusion-tropomyosin, in contrast to the lack of excimer for tropomyosin, indicates that pyrenes in the fusion domain contribute to the excimer fluorescence. Thus, there is a close interaction between the fusion peptides of the globular domain. The decrease of the excimer fluorescence starting at 65 °C suggests that dissociation of the chains of the fusion domain accompanies the unfolding observed by Trp fluorescence.

Electron micrographs of the fusion-tropomyosin molecule show the presence of a globular domain at one end of the rodlike tropomyosin molecule increasing the length of tropomyosin by about 6 nm (Fig. 5). The different size of the globule for different molecules suggests that the globule is shaped like a disc so that its appearance would depend on its disposition on the mica surface. These micrographs indicate: (1) that the fusion peptides at the N-termini of the two tropomyosin chains are in close proximity to each other, interacting to form a globular domain or a pair of closely interacting globular domains; (2) that there is little or no direct interaction between the fusion domain and the main part of the tropomyosin molecule; and (3) a lack of intermolecular association of fusion-tropomyosin at low protein concentration.

Thus, the N-terminal domain in fusion-tropomyosin appears to be stabilized by a coupling interaction with the fusion domain. Further evidence for the influence of the fusion domain on the stability of the N-terminal domain was obtained by comparing the helix unfolding temperatures in different solvents that affect the stability of the tropomyosin helix and the fusion domain to different extents. In the presence of 1 M guanidine-HCl (GuHCl) the fusion domain of fusion-tropomyosin was

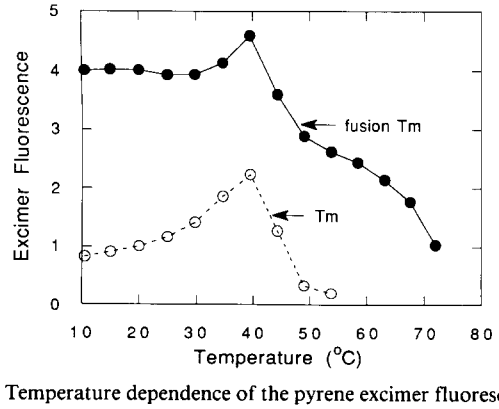

Fig. 4. Temperature dependence of the pyrene excimer fluorescence of pyrene iodoacetamide-labeled fusion-tropomyosin and tropomyosin. The relative intensities of the two profiles are arbitrary. [Tm] = [fusion Tm] = 0.05 mg/mL in solvent of Figure 1; λ_{ex} = 340 nm; λ_{em} = 485 nm.

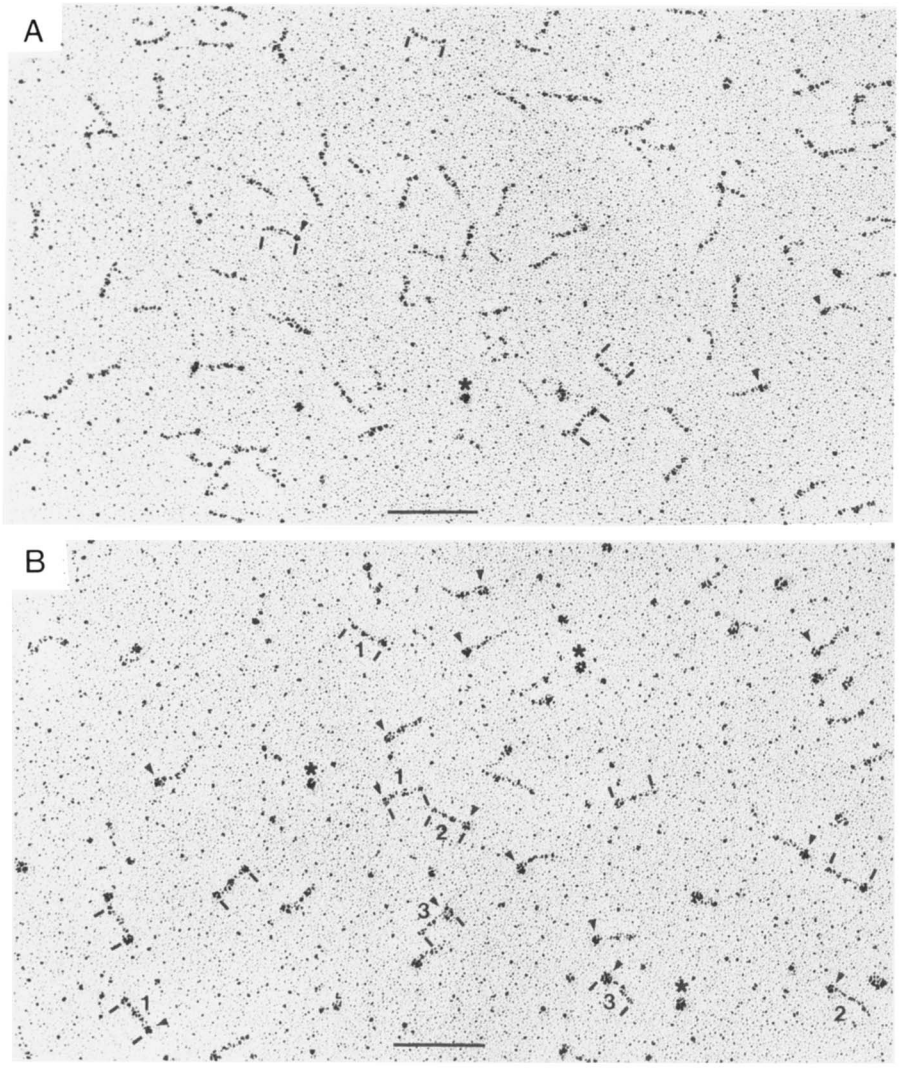

Fig. 5. Comparison of rotary shadowed electron micrographs of fusion-tropomyosin (**B**) with tropomyosin (**A**). The individual rodlike molecules of 40 nm and 46 nm, for tropomyosin and fusion-tropomyosin, respectively, are marked at the ends (short lines). The fusion-tropomyosin molecules have a small globule attached to one end of the rod (arrowheads), accounting for the length increase. The globule sizes vary (marked by 1, 2, 3). Occasionally, small globules are seen at one end of tropomyosin (arrowheads in A) and a few globular objects not associated with rods are seen (asterisks). Magnification: ×120,750; bars indicate 100 nm.

selectively destabilized as indicated by a shift of the Trp fluorescence transition temperature of fusion-tropomyosin from >65 °C to 52 °C. This concentration of denaturant was not sufficient to affect the tropomyosin helix because the main transition of tropomyosin was not changed. In 1 M GuHCl, the unfolding of the helix of fusion-tropomyosin showed only one transition at 46 °C, indicating that the ability of the fusion domain to stabilize the N-terminal domain is reduced when the fusion domain is selectively destabilized. In the presence of 30% glycerol, the main transition temperature of tropomyosin increased 13 °C, but the transition temperature of the fusion domain only increased 5 °C. The transition temperature of the C-terminal domain of fusion-tropomyosin increased 13 °C, as great as that of tropomyosin, whereas the transition temperature of the N-terminal domain of fusion-tropomyosin increased only 8 °C, slightly more than the increase of the transition temperature of the fusion domain, indicating the selective influence of the fusion domain on the N-terminal domain. Thus, these perturbation studies provide evidence for the influence of the fusion domain on the stability of the N-terminal domain.

Discussion

The thermal unfolding of fusion striated muscle $\alpha\alpha$-tropomyosin studied by CD and fluorescence is schematically illustrated in Figure 6. There are four unfolding transitions labeled by the approximate transition temperatures, T_M, T_C, T_N, and T_F. The first three transitions involve tropomyosin helix unfolding of the middle and the C- and N-terminal domains (Fig. 1), and the fourth transition unfolds the nonhelical fusion domain, composed of interacting fusion peptides (Figs. 3, 4). The first transition is a broad unfolding pretransition starting at about 30 °C resulting in the loss of at least 18% of the α-helix. Pyrene excimer fluorescence data (Fig. 4) indicated that this unfolding region includes Cys 190. The transition at 46 °C (T_C), where at most 36% of the helix is

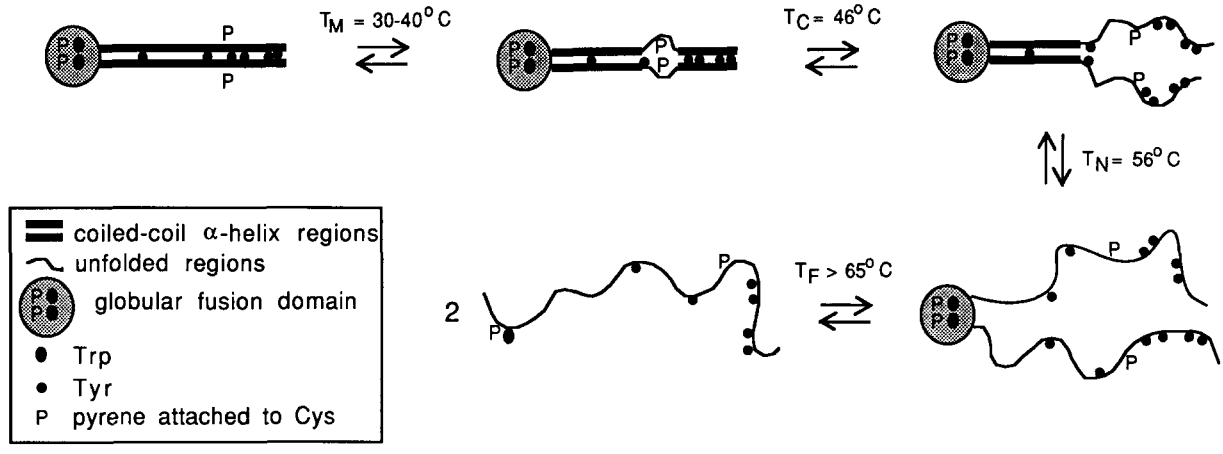

Fig. 6. Schematic diagram of the thermal unfolding of fusion-tropomyosin. Note the approximate location of pyrene, Tyr, and Trp, and the approximate transition temperatures of the unfolding of each domain. Thick lines indicate α-helix; thin lines indicate unfolded regions.

lost, involves the C-terminal domain because the Tyr CD signal, from residues in the C-terminal half, is lost in the same transition (Fig. 2). The middle region is also involved in this transition as indicated by the decrease in excimer fluorescence from pyrenes at Cys 190. Thus, the average distance between pyrenes at Cys 190 increases in the 46 °C transition although the two chains continue to interact at the fusion and N-terminal domains. The 56 °C transition (T_N), where about 46% of the helix is lost involves the remaining N-terminal domain, in agreement with the lack of affect on the Tyr CD signal in this temperature range. The transition above 65 °C (T_F) is associated with the unfolding of the fusion domain. Because the fusion domain is nonhelical, no changes in the ellipticity at 222 nm were observed The unfolding of the fusion domain, however, was monitored by tryptophan and pyrene fluorescence changes, which both showed a transition above 65 °C (Figs. 3, 4).

From the approximate losses of helix in the two main transitions of fusion-tropomyosin, the N-terminal domain is estimated to consist of $0.46 \times 284 = 131$ residues (residues 1–131), and the C-terminal domain is estimated to consist of no more than $0.36 \times 284 = 102$ residues (residues 182–284). The difference, residues 132–182, can then be assigned to most of the unstable middle. This assignment is in good agreement with the region sensitive to proteolytic enzyme digestion (Ueno, 1984). The stabilization by the fusion domain does not extend beyond the N-terminal domain of the molecule, since the pretransition and the C-terminal transition midpoints for fusion-tropomyosin are very similar to the transition midpoints of tropomyosin, not containing the fusion peptide. Thus, the unstable middle of the molecule acts as a boundary separating the C and N domains.

In the case of tropomyosin lacking the stabilizing fusion domain, only one main CD transition is seen with a similar midpoint as the C-terminal domain of fusion-tropomyosin. It thus appears that the N- and C-terminal regions have similar stabilities. However, further studies are necessary to clarify if these two regions unfold as two independent domains.

Questions exist regarding the thermal unfolding pretransition of tropomyosin that occurs in the 30–40 °C temperature range. Does the pretransition occur for reduced uncross-linked striated tropomyosin as it does for disulfide cross-linked tropomyosin? Which region of the molecule is involved? What is the nature of the partially unfolded intermediate? The pretransition was originally observed for disulfide cross-linked tropomyosin (Lehrer, 1975) with CD (Lehrer, 1978) but it was also subsequently observed for reduced tropomyosin with CD and for Cys-labeled tropomyosin with fluorescence and ESR studies (Graceffa & Lehrer, 1980, 1984; Betteridge & Lehrer, 1983). Although the helix pretransition is not as prominent for reduced tropomyosin due to overlapping with the main transition, the probe studies showed that the partial unfolding involves a region that includes Cys 190 and proteolytic digestion studies on unlabeled and cross-linked tropomyosin indicated that the unstable region was approximately located between residues 132 and 183 (Ueno, 1984). CD studies on cross-linked tropomyosin also showed that the Tyr CD signal only changed in the main transition (Lehrer, 1986), verifying the lack of involvement of the C-terminal half in the pretransition. An alternate proposal has been made, in disagreement with the experimental evidence, however, in which the C-terminus of disulfide cross-linked tropomyosin unfolds from the ends toward residue 190 (Skolnick & Holtzer, 1986). However, the localization of the unstable region in the middle of fusion-tropomyosin explains the development of an unfolded boundary, which then allows for the independent unfolding of the N- and C-terminal do-

mains of fusion tropomyosin observed in this work. Early fluorescence studies on tropomyosin labeled with a dansyl fluorophor at Cys 190 gave information about the conformation of the intermediate. Spectral and lifetime studies showed that the dansyl environment becomes more hydrophobic in the pretransition than in the native and completely unfolded states suggesting that the pretransition intermediate is not in a random coil conformation (Betteridge & Lehrer, 1983).

The stabilization of the N-terminal unfolding domain by the fusion domain in fusion-tropomyosin is the result of the strong interaction of the two fusion peptides acting as a cross-link at the ends of the tropomyosin molecule. There is evidence that the NS1 virus protein, from which the fusion peptide is derived, self associates following viral infection (Morrongiello & Dales, 1977; Shaw & Compans, 1978). Recent studies consider domain stabilization via an interdomain interface of interaction with the major part of the coupling free energy coming from ΔH^0 (Brandts et al., 1989; Ramsay & Freire, 1990). For a long linear coiled-coil molecule such as tropomyosin, no obvious structural domains are present, nor do the electron micrographs show an interface between the fusion domain and the tropomyosin part. The major stabilization by the fusion cross-link can arise, however, from a reduction in ΔS^0 of unfolding. Such a mechanism was used to explain the stabilization of tropomyosin produced by the introduction of an interchain disulfide bond at Cys 190 (Lehrer, 1978). Disulfide bond stabilization in other tropomyosins has been observed (Holtzer et al., 1990). For a two-chain molecule such as tropomyosin, an interchain cross-link would reduce the number of unfolded configurations (Kauzmann, 1959) due to the prevention of subunit dissociation that accompanies unfolding (Pont & Woods, 1971; Holtzer et al., 1983; Lehrer & Stafford, 1991). Disulfide cross-links and loops connecting helices have been introduced to increase stability in leucine zipper coiled-coils (O'Shea et al., 1989) and in designed helix bundle proteins (Reagen & DeGrado, 1988; Hecht et al., 1990). The stabilization of the helix in the basic region of leucine zipper coiled-coils (O'Neil et al., 1990; Shuman et al., 1990; Talanian et al., 1990; Weiss, 1990) due to the strong interaction of its ends with DNA may be the result of the cross-link formed. The stabilization of a coiled-coil helix by such an interaction will depend on the difference in the entropy of the unfolded cross-linked chain compared to the unfolded un-cross-linked chain.

The stabilization by the fusion peptide is modified when unstable regions are introduced into tropomyosin in the case of striated-smooth muscle chimeras and for deletion mutants (Ishii et al., 1990). These studies will provide information on the mechanism of the transmission of the strong interaction at the N-terminus through the coiled-coil helix.

Although the relationship of the tropomyosin sequence to possible functional domains remains unclear, some progress has been made in showing the special importance of the C-terminal exon coding region in determining differences in actin binding properties (Cho & Hitchcock-DeGregori, 1991) and thermal stability of recombinant chimeric striated and smooth $\alpha\alpha$-tropomyosin (Ishii et al., 1990). The stabilization of domains in coiled-coil molecules, by localized interaction with other proteins, offers a means to transfer functional information over long distances, which may be important in the generation and regulation of muscle contraction (Zot & Potter, 1987).

Materials and methods

Fusion-tropomyosin was prepared from full-length cDNA clones of rat striated muscle obtained from Dr. B. Nadal-Ginard (Ruiz-Opazo & Nadal-Ginard, 1987), subcloned, and expressed in *Escherichia coli* using a rat striated muscle α-tropomyosin cDNA (gift of Dr. B. Nadal-Ginard; Ruiz-Opazo & Nadal-Ginard, 1987) that was cloned in pas ΔEH801 (Young et al., 1983) to produce a fusion protein with 80 amino acids of influenza virus NS1 on the amino terminus (Heald & Hitchcock-DeGregori, 1988; Cho et al., 1990). It was purified and renatured from GuHCl extracts as described earlier (Hitchcock-DeGregori & Varnell, 1990). Rabbit cardiac tropomyosin was prepared and purified as outlined earlier (Betteridge & Lehrer, 1983). The concentration of tropomyosin was determined by UV absorbance with $A_{277\,nm} = 0.24\ (mg/mL)^{-1}\ cm^{-1}$, and the concentration of fusion-tropomyosin was determined by BCA protein assay (Pierce) using tropomyosin as a standard. The proteins were labeled with pyrene iodoacetamide in the presence of GuHCl as described earlier (Ishii & Lehrer, 1990) keeping the concentration of fusion-tropomyosin at 0.1 mg/mL and the salt concentration above 0.5 M to avoid aggregation.

Fluorescence and CD data were obtained on Spex 2/2/2 Fluorolog and Aviv 60DS CD instruments, respectively, as outlined (Ishii & Lehrer, 1990). The temperature dependence of Tyr CD was obtained by averaging intensities for 25 s after 5–15 min equilibration and correcting for background and baseline drift by subtracting the ellipticity at 300 nm at each temperature. Both the ellipticity and the fluorescence values were reversible within experimental error after the heating and cooling cycles.

Electron micrographs were obtained by spraying 10 μg/mL protein in 0.4 M ammonium acetate and 30% glycerol onto mica and dryed at room temperature and examined with a Philips EM 300 electron microscope (Mabuchi, 1990).

Acknowledgments

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