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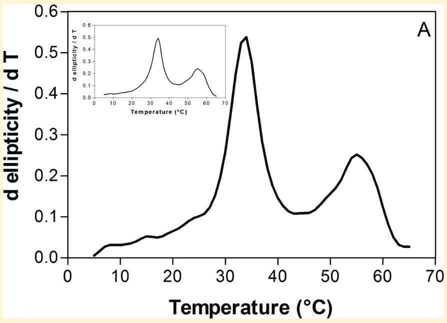
Cold Adaptation of Tropomyosin

Michael Hayley, Tatiana Chevaldina, and David H. Heeley*

Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland A1B 3X9, Canada

ABSTRACT: The conformational stability of unphosphorylated and phosphorylated α,α-striated tropomyosins from rabbit and shark (95% identical sequences) has been investigated. Three additional core positions are occupied by atypical amino acids in the protein from shark: Thr179(d), Ser190(a), and Ser211(a). These changes are thought to have further destabilized most, if not all, of the carboxyl-terminal half of the molecule. Heat-induced unfolding of shark tropomyosin (2 mg/mL, 0.1 M salt, pH 7) as monitored by far-UV circular dichroism is biphasic [$T_{m1} \sim 33$ °C (main), and $T_{m2} \sim 54$ °C] and takes place over a wider temperature span than that of the mammalian protein. The relationship between ellipticity (and excess heat) and temperature is insensitive to the presence in either tropomyosin of covalently bound phosphate. At ~10 mg/mL, the minor endotherm of shark tropomyosin is shifted to ~60 °C and $T_{m2} - T_{m1}$ is increased to 25 °C; otherwise, the results of calorimetry are in agreement with those of circular dichroism. Analyses of cyanogen bromide fragments by far-UV circular dichroism and intact protein by near-UV circular dichroism ($T_m \sim 32$ °C) show that the most stable sizable portion of shark tropomyosin is located within the amino-terminal half of the molecule. These findings illuminate those regions in tropomyosin where flexibility is critical and show that substitutions predicted to be unfavorable in one temperature regime are desirable in another.



Tropomyosin is a rod-shaped protein consisting of two α-helices folded into a coiled coil. It is present in virtually all eukaryotic cell types but is most abundant in striated muscle.[1] The sequence of 284 amino acids[2] contains a repeat in which most of the side chains in the first (a) and fourth (d) positions of a unit of seven amino acids are nonpolar. For the native configuration, these so-called "core" residues are positioned at the interface of the two chains, thereby stabilizing the coiled coil, while polar and charged side chains are solvent-exposed.[3−5]

Structural determinations have shown that certain regions of tropomyosin depart from a perfect coiled-coil configuration.[6−13] The presence of atypical (noncanonical) residues in the a and d positions enhances chain flexibility that, in turn, has important ramifications for function.[14−16] The importance of chain flexibility is brought into sharp focus at lower temperatures where thermal motion is restricted. Globular proteins from organisms (psychrophiles) that live below 15 °C generally possess fewer ion pairs and a smaller hydrophobic contact area compared to warm-bodied homologues.[17−21] But what of rod-shaped proteins?

Shark skeletal muscle tropomyosin is a useful model for addressing this question. It is an α−α homodimer[22] that is 95% identical in sequence with the counterpart in rabbit (Table 1). The sites of phosphorylation, cyanogen bromide cleavage, charged, and aromatic amino acids are all conserved. The first 130 residues are completely identical with two exceptions. At these sites (residues 45 and 63), the two tropomyosins exchange serine and alanine. Four substitutions are situated between residues 132 and 143, but the most striking difference is a cluster of three core substitutions, specifically, Thr179(a)Ala, Ser190(d)Cys, and Ser211(a)Ala. These are the only changes involving core residues. In each instance, the side chain in the mammalian protein is replaced by one of greater polarity in the shark protein that, as a consequence, loses two core alanines (Table 1). Significantly, these substitutions are near three conserved noncanonical core residues, specifically, 214(d), 218(a), and 221(d). Thus, there is an expanded zone of such amino acids in the α-tropomyosin from shark.

Another aspect of this work concerns the outcome of a covalently bound phosphate group within tropomyosin. The two α-tropomyosins selected for study here are isolated from the natural source as a mixture of phosphorylated and unphosphorylated forms. Research has shown that an ionized phosphate stabilizes the end-to-end interaction of shark tropomyosin,[22] consistent with the site of modification being serine 283.[23−25] There are conflicting viewpoints concerning the structural details of the terminal regions of tropomyosin.[10−12] Because phosphate is destabilizing when located at the carboxyl-terminal end of an α-helix,[26,27] it was of interest to compare the conformational stabilities of the two forms of tropomyosin.

Unfolding of α-tropomyosin from rabbit cardiac muscle[28] and mako shark swimming muscle was monitored using circular dichroism and differential scanning calorimetry under conditions (≥0.1 M salt and pH 7) where the protein is predominantly a monomer of 66000 Da. Shark tropomyosin is demonstrated to be less stable than its mesophilic counterpart over more than half its length. Most, if not all, of the carboxyl-terminal half of the

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Table 1. Summary of Amino Acid Substitutions between Rabbit and Shark α-Striated Muscle Tropomyosins[a]

residue	position	shark	rabbit
45	c	Ala	Ser
63	g	Ser	Ala
132	f	Asn	Ser
135	b	Leu	Gln
138	e	Asp	Glu
143	c	Leu	Ile
157	c	Glu	Asp
179	d	Thr	Ala
188	f	Ser	Gly
190	a	Ser	Cys
211	a	Ser	Ala
212	b	Asp	Glu
247	b	Ala	Thr
284	d	Met	Ile

[a] Sequence information taken from refs 2 and 22. The second column denotes the heptad position. Three core substitutions occur at positions 179, 190, and 211. Note that there is an error in Figure 1 of ref 22. Residues 135, 138, and 143 in rabbit α-tropomyosin are incorrectly aligned with the sequence of shark tropomyosin. The correct alignment appears above.

molecule unfolds across the 30 °C range, consistent with the substitutions described above having tailored the molecule for function at low temperatures. Somewhat surprisingly, shark tropomyosin displays a second cooperative transition (midpoint of ~55 °C) that may arise from the need to maintain integrity in the face of the hyperflexibility in the rest of the molecule.

EXPERIMENTAL PROCEDURES

Tropomyosin was isolated[29] from rabbit cardiac muscle and sections of trunk muscle of mako shark *Isurus oxyrinchus*. The determined amino acid composition of mako shark tropomyosin[30] is virtually identical to what is predicted from the sequence of dogfish shark *Scyliorhinus retifer* skeletal muscle tropomyosin.[22] Protein that had been passed over hydroxylapatite (Bio-Rad) was separated into the phosphorylated and unphosphorylated forms as described previously[22] and verified electrophoretically.[31] Cyanogen bromide cleavage of mako shark tropomyosin was conducted using the method described in ref 32. The digest was chromatographed on hydroxylapatite to yield the CN1A (residues 11−127) and CN1B (residues 142−281) fragments. The column buffer contained 1 M NaCl and 0.01% (w/v) NaN_3. The experiment started at 10 mM sodium phosphate (pH 7.0). The experiment ended at 250 mM sodium phosphate (pH 7.0). The column dimensions were 2.5 cm × 15 cm. The gradient volume was 800 mL. Elution of the amino-terminal fragment preceded that of the carboxyl-terminal fragment. Acid hydrolysates were analyzed on a Beckman model 121 MB amino acid analyzer as described previously[30] to yield protein and fragment concentrations based on alanine content. Protein concentrations were also measured by absorbance based on a content of six tyrosines per chain.

Electronic circular dichroism measurements were recorded on a Jasco-810 spectropolarimeter. Heat-induced unfolding of tropomyosin was effected by a linear temperature ramp while the ellipticity was continuously monitored at either 222 or 280 nm.

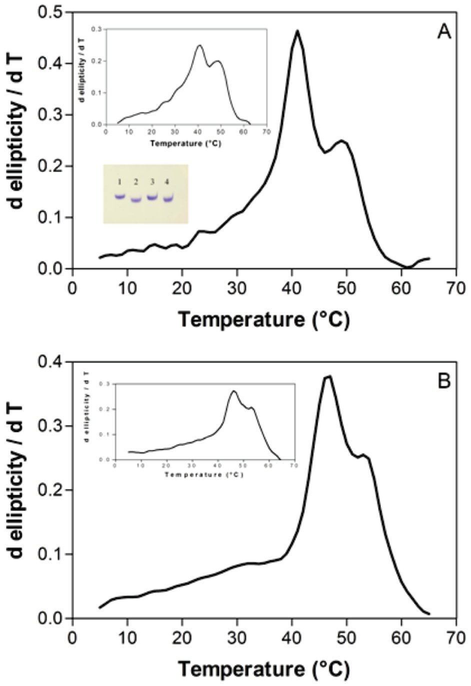

Figure 1. Thermal denaturation of rabbit α,α-tropomyosin at neutral pH as monitored by far-UV circular dichroism. Experimental details are as follows: protein concentration, ~2 mg/mL; light path, 0.1 mm; buffer, 20 mM potassium phosphate, 0.1 or 1.0 M KCl, 1 mM EDTA, and 1−2 mM dithiothreitol (pH 7.00); heating rate, 60 °C/h. Decreasing the heating rate had no bearing on the measured melting temperatures (data not shown). Ellipticity was monitored at 222 nm. The unfolding profiles are presented as the first derivative of the ellipticity change vs temperature. (A) Unphosphorylated tropomyosin in 0.1 M salt, Tms, 41 and 49 °C. (Inset I) Phosphorylated tropomyosin in 0.1 M salt, Tms, 40.6 and 49 °C. (Inset II) Coomassie Brilliant Blue R-250-stained section of an alkaline urea−polyacrylamide gel:[31] lane 1, unphosphorylated tropomyosin before heating; lane 2, phosphorylated tropomyosin before heating; lane 3, unphosphorylated tropomyosin after heating; lane 4, phosphorylated tropomyosin after heating. (B) Unphosphorylated tropomyosin in 1 M salt, Tms, 46.6 and 53.2 °C. (Inset) Phosphorylated tropomyosin in 1 M salt, Tms, 46.2 and 53 °C.

The temperature was controlled by a CTC-345 circulating water bath. Different heating rates from 15 to 80 °C/h had no observable effect on the transition temperature that, in this study, was obtained from the first derivative of the progress curve. In most instances, a heating rate of 60 °C/h was used. Unfolding was also studied using a Microcal MC-2 high-sensitivity scanning calorimeter (Microcal Inc.). Heat capacity profiles were obtained by increasing the temperature at a rate of 30 °C/h. Samples destined for thermal unfolding were dialyzed in the cold against the reference buffer specified in the respective legend. For a single sample, at least three cycles of heating, cooling, and reheating were performed. In some of these repeats, a small shift of ≤1 °C in a given endotherm was observed.

RESULTS

Mammalian Tropomyosin. Rabbit cardiac tropomyosin is identical to the main α-isoform in skeletal muscle.[28] In the mature animal, the protein contains ~0.2 mol of covalently bound phosphate per mole.[23] Previous investigations of conformational stability[33−38] did not distinguish between the two forms of

Table 2. Summary of Unfolding Temperatures of Rabbit and Shark α,α-Striated Muscle Tropomyosins[a]

rabbit			shark		
	unPTM	PTM		unPTM	PTM
circular dichroism			circular dichroism		
0.1 M KCl	$T_{m1} = 40.2 \pm 1.07$	$T_{m1} = 40.4 \pm 0.52$	0.1 M KCl	$T_{m1} = 33.5 \pm 0.79$	$T_{m1} = 33.3 \pm 0.58$
	$T_{m2} = 49.2 \pm 0.59$	$T_{m2} = 49.2 \pm 0.40$		$T_{m2} = 54.6 \pm 1.04$	$T_{m2} = 55.4 \pm 0.58$
1.0 M KCl	$T_{m1} = 46.7 \pm 0.14$	$T_{m1} = 45.9 \pm 0.42$	1.0 M KCl	$T_{m1} = 36.2 \pm 0.62$	$T_{m1} = 35.2 \pm 0.60$
	$T_{m2} = 52.9 \pm 0.42$	$T_{m2} = 53.6 \pm 0.78$		$T_{m2} = 57.7 \pm 0.76$	$T_{m2} = 57.3 \pm 0.59$
calorimetry			calorimetry		
0.1 M KCl	$T_{m1} = 43.7 \pm 0.17$	$T_{m1} = 44.2 \pm 0.30$	0.1 M KCl	$T_{m1} = 35.0 \pm 0.75$	$T_{m1} = 34.5 \pm 0.20$
	$T_{m2} = 53.7 \pm 0.30$	$T_{m2} = 52.9 \pm 0.36$		$T_{m2} = 61.6 \pm 0.45$	$T_{m2} = 60.3 \pm 0.20$
0.1 M KCl (pH 6.0)	$T_{m1} = 42.0 \pm 0.25$	$T_{m1} = 42.1 \pm 0.30$	0.1 M KCl (pH 6.0)	N/A	N/A
	$T_{m2} = 65.6 \pm 0.35$	$T_{m2} = 65.2 \pm 0.40$			
1 M KCl	N/A	N/A	1 M KCl	$T_{m1} = 36.0$	$T_{m1} = 36.0$
				$T_{m2} = 54.6$	$T_{m2} = 54.5$
				$T_{m3} = 66.7$	$T_{m3} = 66.0$

[a] Abbreviations: PTM, phosphorylated tropomyosin; unPTM, unphosphorylated tropomyosin. Values are in degrees Celsius. Unless stated, the pH was 7.00. Because of the availability of protein, more determinations were performed on unPTM than on PTM and by using circular dichroism than by using calorimetry. Samples were prepared from freeze-dried protein obtained from at least two batches of acetone powder and analyzed three or four times. The exception is shark TMs in 1 M salt where only one batch of protein was analyzed multiple times. The transition temperature resolution is 1−2 °C.

α-tropomyosin. In this work, samples of fully phosphorylated and unphosphorylated protein were prepared for analysis (Figure 1A, inset).

The results of circular dichroism experiments, which are summarized in Table 2, shall be presented first. Proteins (∼2 mg/mL) were dialyzed in various salt solutions (pH 7.0) containing dithiothreitol. The ellipticity at 222 nm was then monitored continuously while the temperature was linearly increased from 5 to 65 °C. The midpoint (T_m) of a given cooperative unfolding transition is obtained by converting the progress curve to the first derivative. When this is done, two cooperative transitions are observed for rabbit tropomyosin (Figure 1A,B). The transitions are closely spaced in the temperature gradient and not fully resolved from each other.

In the presence of 0.1 M salt, the averaged T_m values (Table 2) for each form of the protein are not significantly different: unphosphorylated tropomyosin, 40.2 and 49.2 °C (Figure 1A); phosphorylated tropomyosin, 40.4 and 49.2 °C (Figure 1A, inset). In the presence of 1 M salt, both transitions climb to higher temperatures, the first being shifted to a greater extent. Again, similar results are obtained with unphosphorylated [46.7 and 52.9 °C (Figure 1B)] and phosphorylated [45.9 and 53.6 °C (Figure 1B, inset)] tropomyosin. The outcome was the same when the experiments were repeated with proteins dissolved in pH 7 HEPES buffer (data not shown). Further, the integrity of the protein is unaffected by the temperature ramp (Figure 1A, inset).

Calorimetry (Figure 2) was conducted with protein solutions at a concentration (∼8 mg/mL) higher than those used for circular dichroism. At pH 7 and in the presence of 0.1 M salt, the heat capacity profiles, which are presented as the first derivative, consist of endotherms at 43.7 (main) and 53.7 °C [unphosphorylated (Figure 2A)] and 44.2 (main) and 52.9 °C [phosphorylated (Figure 2B)]. These data are in close agreement with those reported by Williams and Swenson[37] working with reduced or cysteine-modified α,*β*-tropomyosin isolated from rabbit skeletal muscle. Lowering the pH slightly from neutrality increases the thermal stability of the coiled coil. First demonstrated for paramyosin,[39] this has been attributed to an increased density of ions on positively charged groups.[40,41] Accordingly, at pH 6.0 (0.1 M salt), the major endotherm occurs above 60 °C, and there is evidence of an additional component on the descending limb of the first transition (Figure 2C,D), again in excellent agreement with earlier results.[37] The melting temperatures, discounting the extra component, are 42.0 and 65.6 °C for the unphosphorylated form and 42.1 and 65.2 °C for the phosphorylated form. Thus, using the current methodologies, there appears to be little if any difference between the two forms of rabbit α-tropomyosin.

Shark Tropomyosin. Mako shark skeletal muscle is composed of a single sarcomeric α-type tropomyosin.[22] In the isolated form, the protein contains ∼0.2 mol of covalently bound phosphate per mole,[22] comparable to that of the mature rodent heart.[23,42] The measured amino acid composition[30] closely matches what is inferred from nucleic acid sequencing.[22] The inferred absence of cysteine, which precludes the requirement for a reducing agent in the procedures outlined below, was previously confirmed by amino acid analysis and the inability of shark tropomyosin to form disulfide cross-linked dimers.[30]

Fully phosphorylated and unphosphorylated forms of shark tropomyosin were prepared from acetone powders and subjected to the same investigative procedures as outlined above. No significant difference was apparent in the mean residue ellipticities, $[\theta]_{222}$, of the native proteins, which at 5 °C (0.1 M salt, pH 7) were consistently in the region of −40000 mdeg cm^2 dmol^{-1} (data not shown). Application of a temperature ramp yields two cooperative transitions that are widely separated, by at least 20 °C, in the gradient (Figure 3A). The first transition, which accounts for approximately two-thirds of the total change in signal, is characterized by a T_m of ∼33 °C. At the same time, however, the minor transition is positioned in the mid-50 °C range, beyond that of any structural transition induced in rabbit tropomyosin.

No difference is observed in the unfolding profiles of phosphorylated and unphosphorylated shark tropomyosin. The T_m values (summarized in Table 2) are 33.5 and 54.6 °C for the

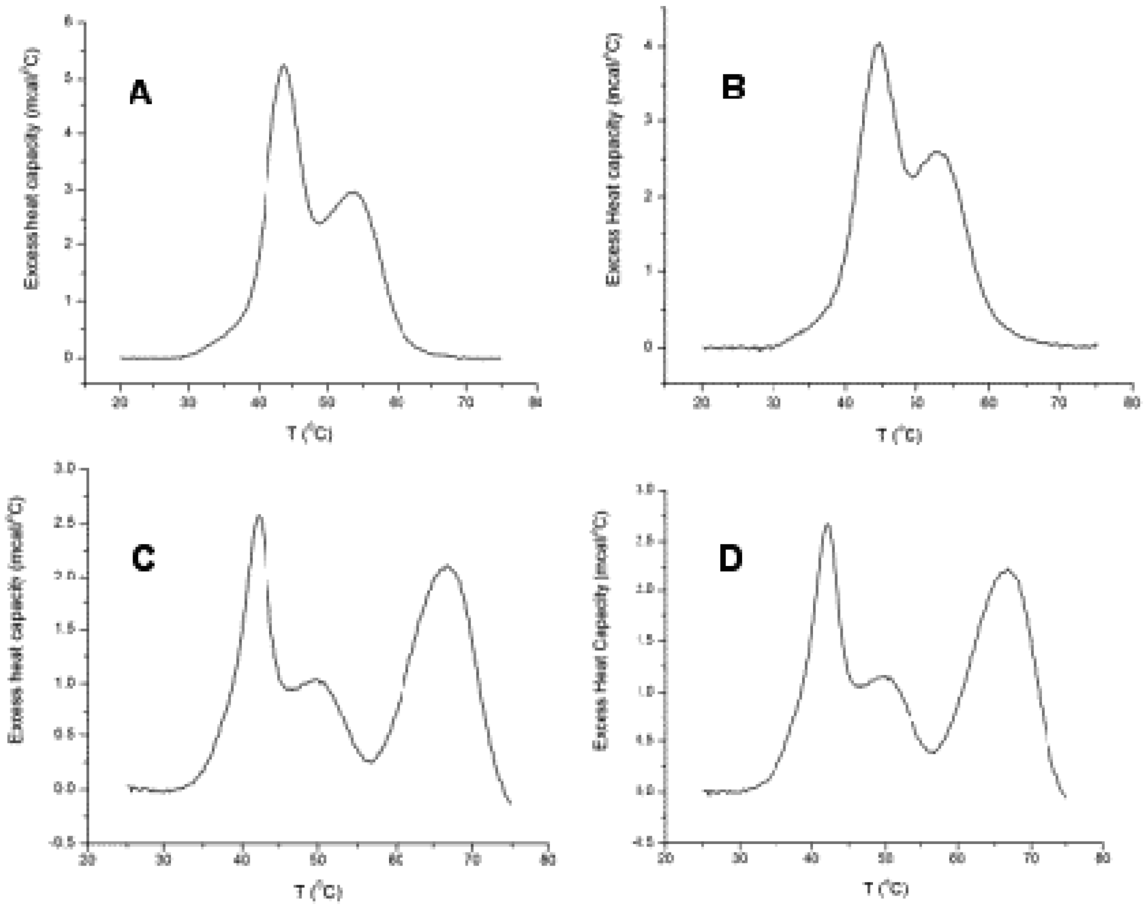

Figure 2. Thermal denaturation of rabbit α,α-tropomyosin in 0.1 M salt as monitored by differential scanning calorimetry. Experimental details are as follows: protein concentration, ~8 mg/mL; buffer, 0.1 M KCl, 1 mM EDTA, 1−2 mM dithiothreitol, and either 10 mM phosphate (pH 7.00, A and B) or 10 mM PIPES (pH 6.00, C and D); heating rate, 30 °C/h. Heat capacity profiles are presented as the first derivative of the progress curve vs temperature. The melting temperatures and enthalpies are indicated. (A) Unphosphorylated tropomyosin at pH 7.0 (44 and 54.3 °C; 268 kcal/mol). (B) Phosphorylated tropomyosin at pH 7.0 (44.5 and 53.3 °C; 215 kcal/mol). (C) Unphosphorylated tropomyosin at pH 6.0 (42.3 and 66.4 °C; 172 kcal/mol). (D) Phosphorylated tropomyosin at pH 6.0 (42.3 and 65.2 °C; 193 kcal/mol). The unfolding transitions were reversible as shown by the performance of multiple runs on the same sample. An additional component is evident in panels C and D at approximately 49 °C in the temperature gradient.

unphosphorylated form and 33.3 and 55.4 °C for the phosphorylated form (Figure 3A and inset). As before, the same result was observed when the experiment was repeated in a non-phosphate pH 7 buffer. Further, phosphodiester bond integrity was confirmed electrophoretically (data not shown). The lower-temperature (main) transition is essentially independent of protein concentration (2−16 mg/mL unphosphorylated protein, 0.1 M salt, pH 7), whereas an 8-fold increase in concentration from 2 mg/mL caused an ~5 °C upward shift in the minor transition (Figure 3B and inset). The wide temperature range over which shark tropomyosin unfolding takes place is also evident at higher salt concentrations (Figure 3C and inset). In this case, both transitions respond similarly (increase in T_m of approximately 2 °C) to the change in ionic strength (Table 2).

The conformational stability of shark tropomyosin was investigated further by the analysis of cyanogen bromide fragments. On the basis of the sequence,[22] chemical cleavage is expected to occur at methionine residues 1, 8, 10, 127, 141, and 281 and yield fragments identical to those obtained with rabbit tropomyosin, namely, CN1A (residues 11−127) and CN1B (residues 142−281). Heat-induced denaturation of the unseparated (total) digest (0.1 M salt, pH 7) produced a prominent transition close to 60 °C, preceded by transitions between 25 and 40 °C (Figure 3D). Two polypeptide-containing fractions were obtained by chromatography on hydroxylapatite, whose identities as CN1A (residues 11−127) and CN1B (residues 141−281) were demonstrated by 280 nm absorbance and electrophoretic mobility (data not shown). On the basis of the $[\theta]_{222}$, these fragments are ~70% helical at 4 °C (0.1 M salt, pH 7), comparable to the values reported for the rabbit tropomyosin fragments.[35] Analysis of fragment CN1A (Figure 3E) gives rise to a sharp transition that is centered near 60 °C (59.3 ± 0.25 °C; $n = 4$), which was noted in Figure 3D. Unfolding of CN1B occurs over a broader range and is complete by the 40 °C mark (Figure 3E, inset). Because the melting temperature of CN1A (Figure 3E) exceeds that of the intact protein, under the same conditions (Figure 3A), fragmentation must have removed a source of destabilization.

Shark tropomyosin was also studied by monitoring unfolding in the aromatic region. On this point, five of the six tyrosines (residues 60, 162, 214, 221, 261, and 267) are located between positions 162 and 267.[22] A plot of $d\theta_{280}/dT$ versus T (Figure 3F

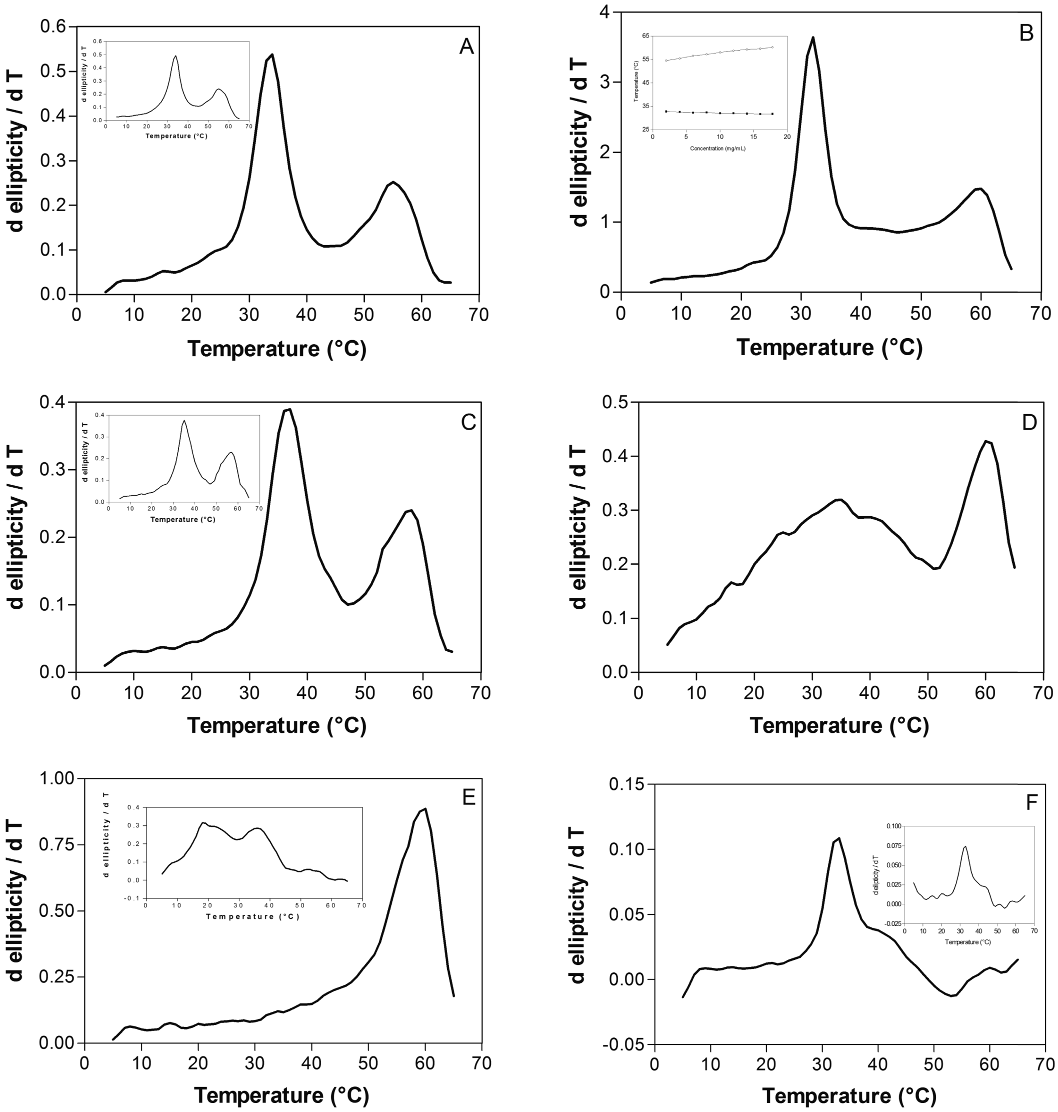

Figure 3. Thermal denaturation of intact and cyanogen bromide-derived fragments of shark α,α-tropomyosin at neutral pH as monitored by circular dichroism. Unfolding was monitored at 222 nm except for panel F, where a wavelength of 280 nm was used. Experimental details are as follows (unless stated otherwise): protein concentration, ~2 mg/mL; light path, 0.1 mm; buffer, 20 mM potassium phosphate, 0.1 or 1.0 M KCl, and 1 mM EDTA (pH 7.00). Other details are given in the legend of Figure 1. The melting temperatures are indicated. (A) Unphosphorylated tropomyosin in 0.1 M salt (33.8 and 55.4 °C). (Inset) Phosphorylated tropomyosin in 0.1 M salt (33.8 and 55.8 °C). (B) Unphosphorylated tropomyosin (16 mg/mL) in 0.1 M salt (31.7 and 59.6 °C). (Inset) Concentration dependence of melting temperature. (C) Unphosphorylated tropomyosin in 1 M salt (36.2 and 57.7 °C). (Inset) Phosphorylated tropomyosin in 1 M salt (35.2 and 57.3 °C). (D) Whole cyanogen bromide digest in 0.1 M salt. (E) CN1A (59.4 °C). (Inset) CN1B. (F) Unfolding monitored by aromatic circular dichroism. Unphosphorylated tropomyosin (32.6 °C). (Inset) Phosphorylated tropomyosin (32.6 °C). Cooling the heat-denatured protein returned the 222 nm ellipticity to ~95% of the starting value. In the inset of panel B, only the more abundant unphosphorylated tropomyosin was used for concentration dependence. In panel D, shark tropomyosin (unphosphorylated) was reacted with CNBr. Fragments were isolated chromatographically. The concentration of the whole digest was determined by absorbance. That of the fragments was determined by amino acid analysis. Samples of CN1A and CN1B (4 °C, 0.1 M salt, pH 7.00) were judged to be ~70% helical on the basis of the molar ellipticity at 222 nm. In panel F, unfolding was monitored at 280 nm: protein concentration, ~12 mg/mL; buffer, 20 mM potassium phosphate, 0.1 M KCl, and 1 mM EDTA (pH 7.00); light path, 2 mm; heating rate, 15 °C/h.

and inset) contains a peak centered at 32.6 °C (0.1 M salt, pH 7), for both fully phosphorylated and unphosphorylated tropomyosin, close to the lower-temperature transition observed during main chain unfolding (Figure 3A and inset). Similarly, when the experiment was repeated with rabbit tropomyosin (unphosphorylated), a transition was observed at ~41 °C (data not shown).

Calorimetric analysis of shark tropomyosin (Figure 4A–D) yields results similar to those of circular dichroism (summarized in Table 2). In the presence of 0.1 M salt (~11 mg/mL protein, pH 7), endotherms of unequal magnitude are observed at ~35 (main) and ~60 °C. Once more, the unphosphorylated and phosphorylated proteins are equivalent (Figure 4A,B). The melting temperature of the minor transition is within 1 °C of what was observed by circular dichroism at a similar protein concentration (Figure 3B), although there is a somewhat larger discrepancy of ~3 °C in the case of the major transition. Increasing the salt concentration to 1 M causes an overall

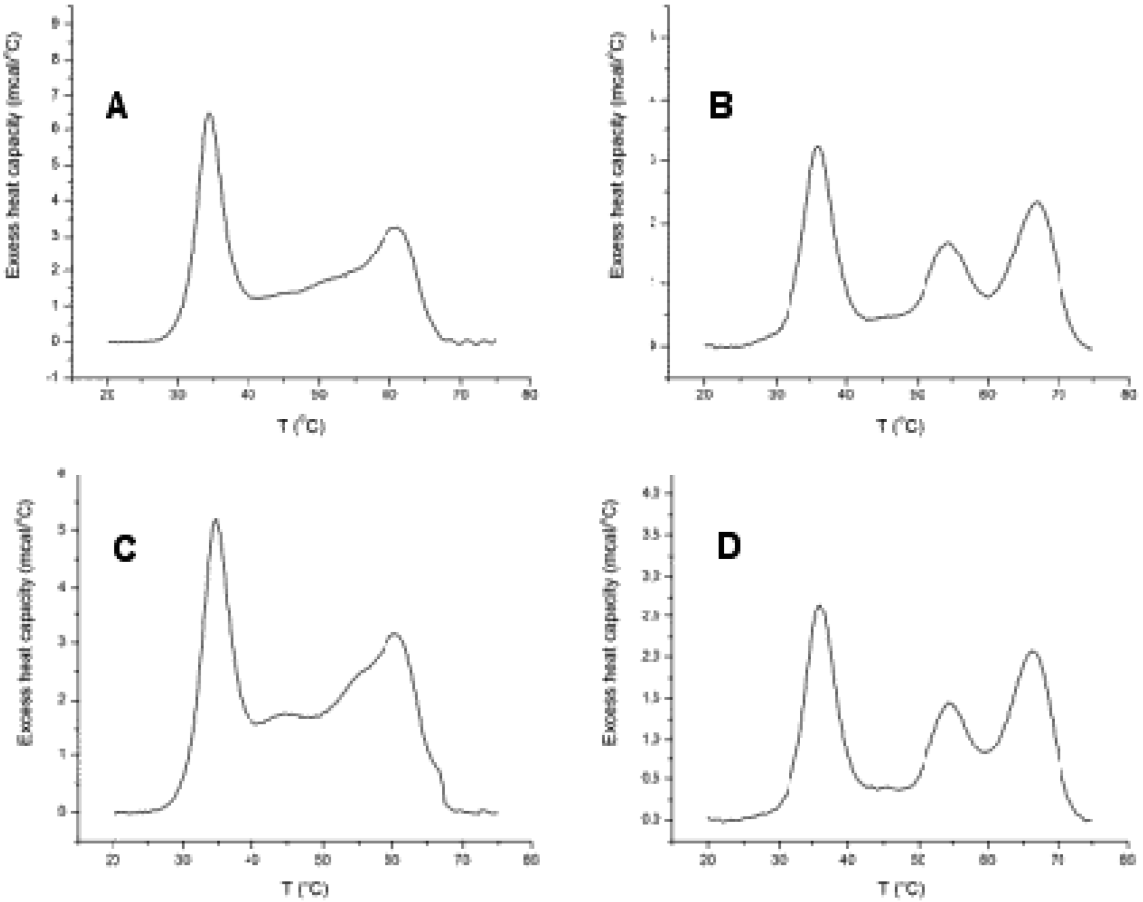

Figure 4. Thermal denaturation of shark α,α-tropomyosin at neutral pH as monitored by differential scanning calorimetry. Experimental details are as follows: protein concentration, ∼11 mg/mL; buffer, 10 mM phosphate, 0.1 or 1.0 M KCl, and 1 mM EDTA (pH 7.00); heating rate, 30 °C/h. Heat capacity profiles are presented as the first derivative of the progress curve vs temperature. The melting temperatures and enthalpies are indicated. (A) Unphosphorylated tropomyosin in 0.1 M salt (34.5 and 61 °C; 232 kcal/mol). (B) Phosphorylated tropomyosin in 0.1 M salt (34.7 and 60 °C; 266 kcal/mol). (C) Unphosphorylated tropomyosin in 1 M salt (36.0, 54.6, and 66.7 °C; 163 kcal/mol). (D) Phosphorylated tropomyosin in 1 M salt (36.0, 54.5, and 66 °C; 160 kcal/mol).

increase in stability, and multiple endotherms are clearly indicated (Figure 4C,D). The latter observation can be explained by the more stable portion of shark tropomyosin being composed of elements that are differentially stabilized by salt.

Thus, investigation of shark skeletal α-tropomyosin has detailed a pronounced variation in conformational stability along the length of the molecule, variation greater than what has been demonstrated for the mesophilic counterpart.[35−38] The relative instability of shark tropomyosin, based on the magnitude of the lower-temperature transition (Figures 3 and 4), spans more than 50% of the molecule and must, therefore, include sequences in each half, but the remainder of the protein displays surprising heat resistance.

■ DISCUSSION

Biomolecules from cold-dwelling organisms, out of necessity, have to counteract the problem of restricted thermal motion. A given globular protein family employs its own unique combination of adaptive strategies, but in general, these involve reducing the number of intramolecular interactions.[17−21] Previous research has confirmed that the a and d (core) amino acids are critical to the stability of tropomyosin.[4,5] This point is also demonstrated by the effect that naturally occurring, atypical core amino acids have on the coiled coil. Examples include aspartate 137(d), which enhances the susceptibility to trypsinolysis,[35,43] glutamate 218(a), which increases chain separation,[13] and glutamine 263 and tyrosine 267, which allow the chains to create a V-shaped cavity.[8−10,12] With regard to the second example, the flanking core positions, 214(d) and 221(d), are populated by tyrosines. All of these amino acids (residues 137, 214, 218, 221, 263, and 267) are conserved in shark skeletal α-tropomyosin.

It has long been appreciated that the stability of mammalian tropomyosin is not uniform along its length. Several lines of evidence point to the existence of heightened flexibility in the center of the molecule.[44−48] The importance of this part of tropomyosin is underlined by the documentation of familial hypertrophic cardiomyopathy (FHC) causing mutations (ref 49 and summarized in Figure 1 of ref 50) between residues 172 and 192.[51−53] Interestingly, shark tropomyosin contains three additional core isomorphisms. In each instance, the equivalent amino acid in rabbit is replaced with a hydroxyl-containing one in shark with the loss of two core alanines: Thr179(d)Ala, Ser190(a)Cys, and Ser211(a)Ala (Table 1). Thus, six destabilizing side chains are predicted for residues 179−221. What is striking is that this section of polypeptide (i) straddles the aforementioned FHC mutations and (ii) is juxtaposed on the segment that contains the core glutamate. Given that the core of the coiled coil is the mainstay in terms of stability, it is surely significant that much, if not all, of the carboxyl-terminal half of the psychrophilic molecule is destabilized relative to the mesophilic counterpart. Further, because the lower-temperature transition accounts for more than 50% of the total unfolding (Figures 3 and 4), it must include sequences within the amino-terminal half, probably including aspartate 137. Long-range effects[48] may be the cause or local heterogeneity between residues 132 and 143 (Table 1).

The mako shark is a marauding predator. It maintains a "warm" body for the purpose of bolstering swimming speed.[54,55] However, the relationship between growth temperature and protein stability is not obvious for the following reasons. First, the trunk never reaches 37 °C, yet of the two tropomyosins examined, it is the one from shark that displays the highest T_m (Table 2). Second, the conformational properties of tropomyosin isolated from blue shark, a cold-bodied species, are equivalent to what is presented in Figures 3 and 4 (data not shown). Third, calorimetric analysis (0.1 M salt, pH 7) of tropomyosin from bluefin tuna,[56] another heated-body fish, in this case a teleost, demonstrated two transitions near 30 and 40 °C.[56] No transition was observed above 50 °C. Comparison is complicated by the fact that tuna tropomyosin comprises two isoforms and is a heterodimer, whereas shark tropomyosin is homodimeric. In addition, a larger number of substitutions exist between tropomyosins from tuna and shark than between shark and rabbit. Threonine 179 is conserved, but the heterogeneity (i.e., tuna vs shark) has had implications for some of the core residues, including residues 190 and 211, as well as other features of the molecule. Another complicating factor is the fact that the mechanism of osmotic balance employed by sharks specifically, the synthesis of nitrogen-containing small molecules, has ramifications for protein stability. The actual basis of the relative stability of approximately one-third of shark tropomyosin is also not clear. There may be a requirement for maintaining the integrity of the homodimer given the enhanced flexibility throughout much of the molecule.

Discussion now turns to the other aspect of the investigation, phosphorylation of tropomyosin. Research has shown that an ionized phosphate group can influence α-helix stability in accordance with the sign of the helix dipole.[26,27] Specifically, an ionized phosphate at the amino terminus of a helix results in stabilization, whereas the reverse is true at the other end. However, when investigated by circular dichroism and calorimetry (under ionic conditions where the end-to-end interaction is annulled), both tropomyosins display an apparent insensitivity to the modification. One interpretation of this result is that the region containing the phosphorylation site is already unfolded in the native state,[10,14] although it is questionable whether any change in helical content could, in the first place, be detected above the large signal background.

In summary, the research described herein is another reminder of the acute sensitivity of tropomyosin to changes in its primary structure, those observations pertaining to phosphorylation notwithstanding. The striated muscle α-tropomyosins from shark and rabbit share but a small number of isomorphisms yet exhibit markedly different patterns of unfolding. The distribution of these isomorphisms is informative because it highlights those regions in the protein where flexibility is at a premium. The altered core positions all fall in a region (from the middle of the molecule to a third of the way toward the carboxyl-terminus) that is essential for the structure and function of the thin filament. The importance of this part of tropomyosin, which contains a disease hot spot, is further underlined by its reduced conformational stability (greater flexibility) in a protein from a cold source. In the case of shark skeletal muscle tropomyosin, the basis of the destabilization is the chemical makeup of the coiled-coil interface, an obvious ploy by which to extend coiled-coil function to a lower-temperature regime. As a corollary, it can be stated that amino acid replacements that are deleterious at one growth temperature can be favorable at another.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (709) 864-2036. Fax: (709) 864-2422. E-mail: dheeley@mun.ca.

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