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Biochemical Characterization of the Roles of Glycines 24 and 27 and Threonine 179 in Tropomyosin from the Fast Skeletal Trunk Muscle of the Atlantic Salmon

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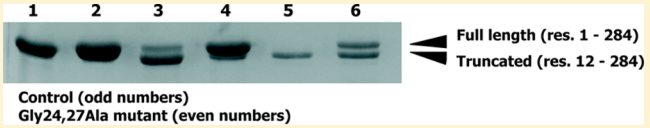
ABSTRACT: Atlantic salmon fast skeletal muscle is composed of an α-type tropomyosin that shares 20 substitutions with a mammalian homologue. Prominent isomorphisms considered to be potentially destabilizing include threonine 179 (core, Ala in rabbit) and a unique pair of glycines, residues 24 and 27. Bacterially expressed mutant tropomyosins were isolated without exposure to elevated temperature or organic solvent. The Thr179Ala mutant is more resistant to heat-induced denaturation (T_m > 40 °C) than the nonmutant control (T_m = 37 °C) as monitored by far-UV circular dichroism [0.1 M salt and 1 mM DTT (pH 7)]. Changing one glycine to alanine has no detectable effect on the T_m versus the control value. An increase of 1 °C is observed for the double mutant (Gly27Ala/Gly24Ala). The preferred site of chymotryptic cleavage of recombinant salmon tropomyosin is between Leu 11 and Lys 12. The rate of the subsequent cleavage at Leu 169, the preferred site in rabbit tropomyosin, is reduced via replacement of Thr 179 with Ala. Thin filaments reconstituted with this mutant display greater Ca(II) induction of the myosin steady-state MgATPase versus control. Glycine-induced local instability is demonstrated by *Escherichia coli* outer membrane protease T (Omp-T) cleavage between Lys 6 and Lys 7. The control is more susceptible to proteolysis at this site than the mutants, which are distinct from each other: control > Gly27Ala > Gly24Ala > double mutant (most resistant to Omp-T). Similarly, the double mutant is comparatively more resistant to cleavage at Leu 11. By inference, the mid- and amino-terminal sections of salmon tropomyosin are intrinsically less stable than those in mammals, suggesting greater flexibility. Neighboring glycines make up a new destabilization motif in tropomyosin.



Tropomyosin[1] is a rod-shaped, regulatory protein located in the thin filament of striated muscle. The amino acid sequence,[2] which lacks proline, contains a strong repeat in which the first ("a") and fourth ("d") residues of a unit of seven are, with a few notable exceptions, populated by nonpolar R groups. In the α-helical conformation, the protein chain self-associates via a knobs into holes interdigitation of the "a" and "d" side chains at the dimer interface.[3] This arrangement is the mainstay for stabilization of a double-stranded, α-helical coiled coil.[4]

Despite the strong short-range repeat, tropomyosin is not uniformly configured from end to end. Rather than being detrimental, such irregularity has important functional ramifications (reviewed in ref 5). The existence of structural imperfections was first suggested by amino acid sequencing whereby certain core positions were found to be occupied by charged [Asp 137(d) and Glu 218(a)] or polar [Tyr 214(d), Tyr 222(d), Gln 263(d), and Tyr 267(a)] residues.[2] Later, variability of pitch, radius, and bending of the molecule was observed in crystals of the full-length protein but at low resolution.[6−9] Finer architectural details were revealed in crystallized sections of tropomyosin.[10−13] This included a misalignment of the side chains of groups of core alanines, a feature that was purported to create bends in the coiled coil thereby allowing it to maintain contact with F-actin. A later study that examined the longitudinal separation between amino acids in the atomic structures revealed that bending of the coiled coil is not only localized to the alanine clusters.[14] Either way, strategically placed instability is part and parcel of the normal structure and function of tropomyosin. As an example, exchanging alanines 74(d), 78(a), and 81(d) with leucine increased the stability but resulted in a decrease in the affinity for actin.[15,16] In this context, there are a growing number of mutations, currently in excess of 50 for all isoforms of tropomyosin (reviewed in refs 17 and 18), that are linked to muscle disease. In the case of the first documented examples, Asp175Asn and Glu180Gly,[19] effects on F-actin binding, susceptibility to tryptic cleavage,[20−23] and regulation[24] were documented.

Imperfections in the coiled coil are predicted to be a feature of tropomyosins from psychrophilic organisms (organisms that live below 15 °C). Proteins from such organisms are generally assumed to contain amino acid substitutions that offset the problem of cold-induced rigidity, an obvious impediment for one having an extensive quaternary structure and a regulatory role that entails shifts in physical position. Such isomorphisms are expected to be missing from mesophilic homologues and to highlight those parts of the molecule where extra flexibility is needed. This investigation utilizes a tropomyosin from Atlantic

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salmon *Salmo salar*, a marine vertebrate that during the winter months lives at single-digit Celsius temperatures.

The fast skeletal trunk muscle (of *S. salar*) is composed of an α-type tropomyosin[25] that is homodimeric and unphosphorylated.[25] The sequence retains Gly 126 and Asp 137, which have been linked to destabilization of the central sections of human α-tropomyosin,[26,27] as well as the other noncanonical core residues listed above. The protein from salmon is more heat-sensitive than rabbit α-striated tropomyosin,[28,29] as would be anticipated, although the sequences share just 20 (mostly conservative) amino acid differences. Of the 20, only one affects the core, namely, Thr 179(d) (Ala in rabbit). This particular isomorphism is conspicuous because the corresponding region of the mammalian homologue is known to be unstable[30,31] and to interact with troponin.[32,33] It also creates a segment encompassing residues 172−216 that is devoid of a core alanine. Further, there are glycines at positions 24(c) and 27(f) that are unique. The proximity of two such helix-disrupting residues[34] in tropomyosin is extremely rare.

The influence of residues 24, 27, and 179 on the conformational stability of salmon tropomyosin has been investigated. Inferred by the outcome of alanine replacement, these isomorphisms produce instability within different parts of tropomyosin. The instability is proposed to combat the constraints imposed by a frigid habitat.

EXPERIMENTAL PROCEDURES

Site-directed mutagenesis of Atlantic salmon fast skeletal tropomyosin cDNA[28] was conducted using the QuikChange Lightning Site-Directed Mutagenesis kit (Stratagene). Alanine was introduced at positions 24 (Gly24Ala), 27 (Gly27Ala), 24 and 27 (double mutant), and 179 (Thr179Ala). Custom-made oligonucleotide primers were obtained from Operon (Huntsville, AL). Mutations were confirmed by sequencing in CREAIT (Memorial University of Newfoundland). Expression vector pTrc99A, containing the tropomyosin cDNA of interest, was transfected into BL21 cells. The cells were grown at 37 °C, and expression was induced using isopropyl β-D-thiogalactopyranoside. Protein isolation was conducted without resorting to elevated temperature or organic solvent. Pelleted cells were dispersed in 0.2 M NaCl, 50 mM MOPS, and 1 mM DTT (pH 7.0) and passed through a French pressure cell at room temperature. The lysate was diluted into more buffer and stirred for 15 min at room temperature upon addition of phenylmethanesulfonyl flouride to a millimolar concentration. The tropomyosin-containing supernatant was subjected to isoelectric and ammonium sulfate-induced precipitations in the cold. The 70% ammonium sulfate pellet was dialyzed in the cold and freeze-dried. A high level of enrichment was achieved by chromatography on Q Sepharose Fast Flow [column dimensions, 2.5 cm × 15 cm; start buffer, 75 mM NaCl, 30 mM Tris, and 1 mM DTT (pH 8); end buffer, 500 mM NaCl; cold room] and hydroxyapatite [column dimensions, 2.5 cm × 15 cm; start buffer, 1 M NaCl, 30 mM sodium phosphate, 1 mM DTT, and 0.01% (mass per volume) NaN_3 (pH 7.0); end buffer, 250 mM sodium phosphate; room temperature]. Crude tropomyosin was dissolved in Q Sepharose Fast Flow start buffer upon addition of phenylmethanesulfonyl flouride. Tropomyosin-containing fractions were loaded directly onto hydroxyapatite. Fractions from the latter were exhaustively dialyzed in the cold and freeze-dried. Approximately 30 mg of tropomyosin (unacetylated, 284 amino acids) was obtained per liter of culture.

Proteolytic digestion of recombinant Atlantic salmon tropomyosins by *Escherichia coli* outer membrane protease T (Omp-T) was performed using whole JM109 cells as an enzyme source.[35] A temperature of either 10 or 25 °C was used to obtain a controlled time course of digestion. Tropomyosin (ε_{280} = 16500 M^{-1} cm^{-1}) was dissolved and dialyzed against assay buffer [0.1 M NaCl, 50 mM sodium phosphate, 5 mM EDTA, and 1 mM DTT (pH 7.0)] in a cold room. Cells for one digestion experiment were prepared as follows. LB broth (10 mL) was inoculated with 100 μL of *E. coli* JM109 cells and incubated overnight at 37 °C while being shaken. The following day, the cells were pelleted and dispersed in 1 mL of assay buffer. Typically, 500 μg of a given tropomyosin (in an equal volume of buffer) was added to the 1 mL of dispersed cells. The reaction mixture was mixed at regular intervals, and at selected times, 100 μL was withdrawn and the cells were pelleted by centrifugation (1 min at 12000 rpm in an Eppendorf Centrifuge 5415 instrument). A portion of the supernatant was then added to an equal volume of SDS sample buffer and boiled without waiting. All of the digests were then analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).[36]

Limited chymotryptic (bovine pancreas, Worthington) digestion was conducted in 0.1 M NaCl, 50 mM NH_4HCO_3, and 1 mM DTT (pH 8.5).[37] A stock solution of chymotrypsin (ε_{280} = 51000 M^{-1} cm^{-1}) was prepared by dialyzing overnight against 2 mM HCl in the cold. Reaction was initiated by addition of the enzyme and stopped with ∼1 μM lima bean trypsin inhibitor (Worthington) dissolved in 20 mM Tris (pH 7.5) followed by freezing. The various samples were then thawed and heated in SDS sample buffer all at the same time.

Circular dichroism measurements were recorded on a Jasco-810 spectropolarimeter. Tropomyosins were dialyzed in the cold against 0.1 M NaCl, 20 mM sodium phosphate, and 1 mM DTT (pH 7). Unfolding was induced by heating the protein (∼2 mg/mL) from 5 to 65 °C in jacketed cells with a light path of 0.1 mm while recording the 222 nm ellipticity every 0.2 °C. The temperature, controlled by a CTC-345 circulating water bath, was increased at a rate of 60 °C/h. A normalized unfolding profile was obtained by taking the average of the first five ellipticity readings in the temperature gradient (i.e., from 5 to 6 °C) as 100% (θ start) and that of the last five (i.e., from 64 to 65 °C) as 0% (θ end) and applying the following formula, where θ_T is the ellipticity at temperature T:

$$(\theta_T - \theta \text{ end})/(\theta \text{ start} - \theta \text{ end}) \times 100$$

Measurement of the steady-state rate of MgATP hydrolysis was performed under conditions far removed from V_{max}. Assay buffer consisted of 30 mM NaCl, 10 mM HEPES, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 0.01% (mass per volume) sodium azide (pH 7.10) at 20 °C. Proteins (with the exception of tropomyosin, all isolated from rabbit skeletal muscle) were dialyzed overnight in the cold against the buffer described above. The various assay constituents were combined one at a time in the following order: buffer > F-actin > tropomyosin > troponin > 2 mM $MgCl_2$ > either 1 mM EGTA (pCa > 7) or 0.5 mM $CaCl_2$ (pCa < 4). A mole ratio of tropomyosin and troponin to F-actin of 4/7 was used. After being gently mixed, the mixture was incubated for 30 min at 4 °C before being transferred to a 20 °C bath. Myosin-S1A1 was then added, and the reaction was initiated by 2 mM ATP (from a 0.1 M stock set to neutrality). The amount of inorganic phosphate was

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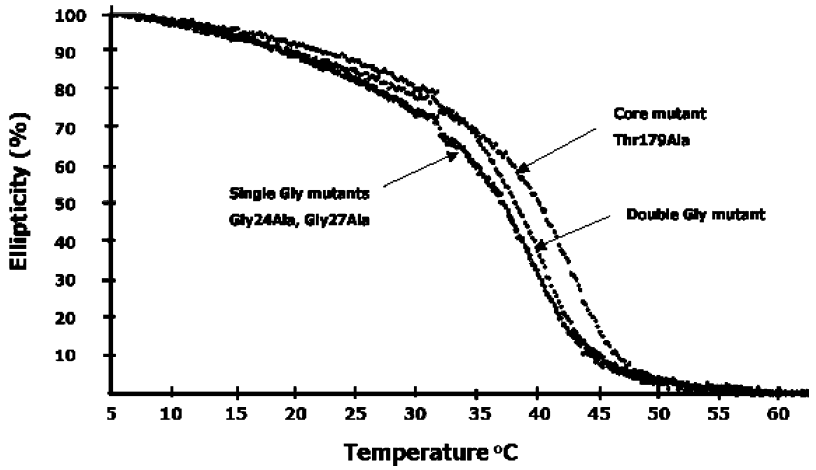

Figure 1. Comparison of the normalized unfolding profiles of recombinant (nonmutant and mutant) Atlantic salmon fast skeletal tropomyosins. Bacterially expressed tropomyosins were dissolved and dialyzed in 0.1 M KCl, 20 mM potassium phosphate, 0.01% sodium azide, 1 mM EGTA, and 1.5 mM DTT (pH 7.0) in a cold room. The samples (~2 mg/mL) were inserted into a cylindrical 0.1 mm path length cell and exposed to a linear temperature gradient from 5 to 65 °C, at a rate of 1 °C/min. Starting mean residue ellipticities (5 °C) were typically 32000 deg cm^2 $dmol^{-1}$. Approximately 10% of the 222 nm signal remained at 65 °C. The unfolding profiles presented above were selected from a number of experiments. The normalized ellipticities for the two single-glycine mutants lie on the same curve. The midpoints of the profiles occur at 37.1 °C (Gly24Ala), 37.3 °C (Gly27Ala), 38.3 °C (Gly24Ala/Gly27Ala, double mutant), and 40.2 °C (Thr179Ala). Averaged melting temperatures, including that of the control tropomyosin, that were obtained from several determinations are listed in Table 1.

determined colorimetrically[38] in aliquots (of 90 μL) taken at five different times.

RESULTS

Thermal Unfolding. The melting temperatures, T_{ms}, of recombinant salmon tropomyosins were obtained from the midpoint of the 222 nm ellipticity versus temperature relationship. It is important to note that the proteins were not exposed to elevated temperature or organic solvent at any time during their isolation. Experiments were conducted in a pH 7 buffer containing 0.1 M neutral salt and reducing agent. Under these conditions and at 5 °C, no significant difference (to within protein concentration error) is evident in the mean residue 222 nm ellipticities of the various tropomyosins. Values are in the region of −32000 deg cm^2 $dmol^{-1}$. Unfolding was elicited by application of a linear temperature ramp. The results of individual experiments are presented in Figure 1. Averaged T_{ms} values from multiple experiments are listed in Table 1.

Identical unfolding profiles are observed for the two single glycine-for-alanine mutants. Further, the melting temperature, T_m, of ~37 °C is no different from that of the control (Table 1). In the case of the double mutant (both glycines replaced with alanine), the midpoint is shifted slightly upward in the temperature gradient (T_m ~ 38 °C). In our hands, a 1 °C difference in T_m is on the borderline of detection. By contrast, significant stabilization is observed in the case of the Thr179Ala mutant. This mutant displays the highest T_m, ~41 °C, of those of the tropomyosins tested (Figure 1). The averaged melting temperatures (Table 1) are 37.0 ± 0.81 °C for the control, 36.9 ± 0.12 °C for Gly24Ala, 37.3 ± 0.33 °C for Gly27Ala, 38.1 ± 0.73 °C for Gly24Ala/Gly27Ala, and 40.7 ± 0.97 °C for Thr179Ala. Thus, threonine (the naturally occurring amino acid in salmon) at position 179 is inferred to be destabilizing relative to alanine (naturally occurring in rabbit). Circular dichroism analysis[29] of the true (acetylated) wild-type protein revealed a major transition (T_m = 38 °C) and a minor pretransition (T_m = 24 °C). The major transition is in line with that of the recombinant version (Table 1). The authors do not have an explanation at this time regarding the apparent absence here of the low-temperature transition. To restate an earlier

Table 1. Average Melting Temperatures of Recombinant (nonmutant and mutant) Salmon Fast Skeletal Tropomyosins and Ca(II) Induction of Regulated Actomyosin-MgATPase[a]

	average melting temp (°C)	Ca(II) induction of regulated actomyosin MgATPase
nonmutant (control)	37.0 ± 0.81 (n = 8)	0.185 s^{-1} (pCa < 4)
		0.028 s^{-1} (pCa > 7)
		6.6-fold
Gly24Ala	36.9 ± 0.12 (n = 3)	nd
Gly27Ala	37.3 ± 0.33 (n = 8)	nd
Gly24Ala/ Gly27Ala	38.1 ± 0.73 (n = 8)	0.206 s^{-1} (pCa < 4)
		0.028 s^{-1} (pCa > 7)
		7.4-fold
Thr179Ala	40.7 ± 0.97 (n = 8)	0.260 s^{-1} (pCa < 4)
		0.028 s^{-1} (pCa > 7)
		9.3-fold

[a]The melting temperature corresponds to the midpoint of the normalized unfolding profile of a sample of tropomyosin that had not been exposed to heat at any time in its history. More than one batch of each tropomyosin was so analyzed, except for the Gly24Ala mutant. Multiple samples from a given batch were analyzed. n is the total number of unfolding experiments performed. Regulated actomyosin steady-state MgATP hydrolysis, expressed as specific activity, was measured at an ionic strength of ~50 mM: 30 mM NaCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 0.01% (mass per volume) sodium azide, 10 mM HEPES (pH 7.1), 1 mM EGTA (pCa > 7) or 0.5 mM $CaCl_2$ (pCa < 4), and 2 mM MgATP. Myosin-S1A1 (4 μM). Reconstituted thin filaments, 4 μM F-actin, 2.28 μM tropomyosin, and 2.28 μM troponin. The temperature was 20 °C. The contribution of myosin-S1 alone, 0.03 s^{-1}, has been deducted. Measurements at pCa < 4 varied by 10% (n = 2).

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point, elevated temperature or organic solvent was not used in any of the protein isolation steps.

Regulation. Thin filaments were prepared with proteins from rabbit skeletal muscle and one of the following tropomyosins: nonmutant, Thr179Ala mutant, or Gly24Ala/Gly27Ala (double mutant). To ensure incorporation of recombinant unacetylated tropomyosin, experiments were conducted in 30 mM NaCl and 3 mM free $MgCl_2$ (ionic strength of ~50 mM) and at a mole ratio of regulatory protein to F-actin of 4/7. Using 4 μM myosin-S1 and 4 μM thin filaments [with EGTA (pCa > 7)], the steady-state rate of MgATP hydrolysis at 20 °C (0.06 s^{-1}) is approximately 2-fold that of S1 alone (0.03 s^{-1}) and does not vary with the tropomyosin constituent (Table 1). A difference is evident between Thr179Ala mutant- and control-containing thin filaments at low pCa. Thin filaments [with Ca(II) (pCa < 4)] reconstituted with the mutant produce ~40% greater activation [0.26 s^{-1} vs 0.18 s^{-1} (Table 1)]. Under the same conditions, the double mutant is not significantly different from the control.

Limited Proteolysis. Limited digestion with chymotrypsin was used to probe for local instability in the Thr179Ala mutant. While this mutation does not result in a unique fragmentation pattern, it does lead to a change in proteolytic susceptibility (Figure 2A). The initial site of chymotryptic cleavage in rabbit α-tropomyosin is Leu 169,[37] near the mutation at position 179. However, at 37 °C, both the mutant and the control are rapidly (within <10 min) degraded to a large fragment (labeled A) that migrates slightly ahead of the intact protein (Figure 2A), indicative of removal of a small flanking peptide. Edman-based analysis (11 cycles) of an electroblotted sample of fragment A matches residues 12–22 of the published sequence:[25] Lys-Leu-Asp-Lys-Glu-Asn-Ala-Leu-Asp-Arg-Ala. Thus, the initial site of chymotrypsin cleavage of recombinant salmon tropomyosin is between residues Leu 11 and Lys 12.

With continued incubation at 37 °C, fragment A gives rise to three major subfragments (B, ~25 kDa; C, ~20 kDa; and D, ~15 kDa) that are evident in the gel lanes corresponding to longer reaction times. The amino-terminal sequences of B and C are identical to that of fragment A [Lys-Leu-Asp-Lys-Glu-Asn (six cycles)], but the first six residues of fragment D are Val-Ile-Ile-Glu-Ser-Leu. This sequence matches residues 170–175[25] and demonstrates proteolysis at Leu 169. Judging from the electrophoretic mobility (Figure 2A), it is probable that fragment D (~15 kDa) extends all, or most, of the way to the carboxy-terminal end. It appears, therefore, that C (residues 12–169) and D arise from the cleavage of A at Leu 169. The origin of fragment B was not investigated further.

An additional observation arising from the chymotrypsin experiment is that fragment A is more resistant to further breakdown when alanine occupies position 179 rather than threonine. For example, after 20 min only a trace of control (Thr 179 containing) fragment A remains (Figure 2, lane 7), and it is not detected at 30 min (Figure 2A, lane 9). By contrast, A is still prominent at these times in the case of the core mutant (Figure 2A, lanes 8 and 10). This observation is consistent with the mutation having stabilized the middle of the molecule.

The fact that the primary site for chymotrypsin is between Leu 11 and Lys 12 is clearly illustrated by conducting the digestion at 10 °C (Figure 2B). Both the control and the Thr179Ala mutant are rapidly amino-terminally truncated, but fragment A is stable over the same incubation period that was used in Figure 2A. Interestingly, the double mutant (Gly24Ala/Gly27Ala) is noticeably resistant to the action of chymotrypsin

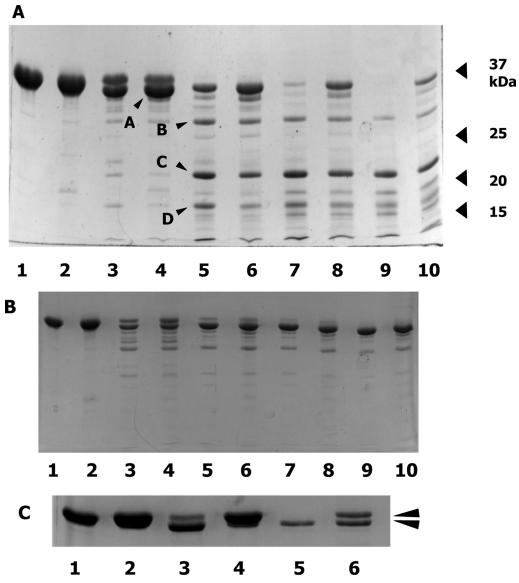

Figure 2. Limited chymotryptic digestion of recombinant (nonmutant and mutant) Atlantic salmon fast skeletal tropomyosin. (A) SDS–PAGE analysis of the time course of chymotrypsin digestion of the Thr179Ala mutant and control (nonmutant) tropomyosins at 37 °C. The two tropomyosins were digested side by side. The buffer consisted of 50 mM NH_4HCO_3, 0.1 M NaCl, and 1 mM DTT (pH 8.5). The enzyme/substrate mole ratio was 1/500. The reaction was started by addition of enzyme and terminated by addition of lima bean trypsin inhibitor and freezing until the sample was prepared for electrophoresis. Control (nonmutant): lanes 1, 3, 5, 7, and 9. Thr179Ala mutant: lanes 2, 4, 6, 8, and 10. No chymotrypsin: lanes 1 and 2. The ensuing lanes correspond to increasing times of digestion, 2, 10, 20, and 30 min. The positions of 15, 20, 25, and 37 kDa standards (Bio-Rad, 161-0363) are indicated in the right margin. Duplicate 12% (mass per volume) polyacrylamide gels were run. One was stained in Coomassie Brilliant Blue R-250 (presented). The other was electroblotted onto polyvinylidine difluoride. Fragments A–D (marked with chevrons) were analyzed by Edman-based sequencing: A Lys-Leu-Asp-Lys-Glu-Asn-Ala-Leu-Asp-Arg-Ala (11 cycles), B Lys-Leu-Asp-Lys-Glu-Asn (six cycles), C Lys-Leu-Asp-Lys-Glu-Asn (six cycles), and D Val-Ile-Ile-Glu-Ser-Leu (six cycles). Because the banding patterns (mutant vs control) are identical, the internal cleavage sites are assumed to be the same and the investigators did not sequence the same fragment from different tropomyosins. (B) Time course of chymotrypsin digestion of Thr179Ala mutant and control (nonmutant) tropomyosins at 10 °C. Conditions were as described for panel A except that a lower temperature was used and the enzyme/substrate mole ratio was 1/250. Control: lanes 1, 3, 5, 7 and 9. Thr179Ala mutant: lanes 2, 4, 6, 8, and 10. No chymotrypsin: lanes 1 and 2. The ensuing lanes correspond to increasing digestion times, 2, 10, 20, and 30 min. As in panel A, the entire Coomassie-stained polyacrylamide gel is shown but at a lower magnification. (C) Time course of chymotryptic digestion of Gly24Ala/Gly27Ala (double mutant) and control (nonmutant) tropomyosins. The temperature was 25 °C. The enzyme/substrate mole ratio was 1/250. Control: lanes 1, 3, and 5. Double mutant: lanes 2, 4, and 6. No chymotrypsin: lanes 1 and 2 (5 min, lanes 2 and 4; 30 min, lanes 5 and 6). Only the section of the Coomassie-stained polyacrylamide gel containing intact (upper chevron) and amino-terminally truncated (lower chevron) tropomyosin is shown. The amount of intact tropomyosin (upper band) remaining was estimated by densitometry (using AlphaView version 3.40). Lane 3, 33% of total (lower band 66%); lane 4, 80%; lane 5, 0%; lane 6, 51%.

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at this site (Figure 2C). Whereas the control is more than 50% truncated after 5 min at 25 °C (Figure 2C, lane 3), the double mutant is essentially intact (Figure 2C, lane 4). Further, while none of the full-length control remains after 30 min (Figure 2C, lane 5), the double mutant comprises a 50/50 mixture (Figure 2C, lane 6).

The previous finding was investigated further using bacterial outer membrane protease T, Omp-T, which hydrolyzes the sixth peptide bond in tropomyosin, between Lys 6 and Lys 7.[35] An incubation temperature of 10 or 25 °C was employed to obtain a manageable rate of digestion. Comparison of the Gly24Ala mutant versus the control is presented in Figure 3A. The entire (nonsectioned) stained gel is shown to demonstrate that proteolysis has not occurred at more than one site. The only bands that are present on the gel correspond to intact (residues 1−284) and truncated (residues 7−284) tropomyosin.[35] Inspection of Figure 3A reveals a large discrepancy in the extents of cleavage of the two proteins. For example, at the end of the 4 h incubation, the control is largely, if not entirely, proteolyzed but the mutant is largely intact. A similar result is obtained with the other single-glycine mutant (Gly27Ala). In this instance, only a section of the stained gel is presented (Figure 3B). Densitometry (Figure 3C) reinforces the notion that the mutant is substantially more resistant to breakdown than the control (Figure 3C).

The three glycine to alanine mutants were then compared with each other to determine the influence of each glycine on the rate of amino-terminal cleavage. It is evident from the results (Figure 3D,E) that the mutants are not equivalent. Estimates of the amounts of intact protein remaining after 30 min are 55% Gly24Ala (Figure 3D, lane 7), 37% Gly27Ala (Figure 3D, lane 8), and 70% double mutant (Figure 3D, lane 9). At the end of the incubation, less than half of the single-site mutant chains remain intact (35% for Gly24Ala and 20% for Gly27Ala), but the double mutant consists of a 50/50 mixture (Figure 3E). To be clear, the digestions were conducted at the same time (i.e., side by side). Furthermore, the experiment was performed on three separate occasions, and the results were in agreement. Altogether, the susceptibility to Omp-T decreases in the following order: nonmutant (least resistant) > Gly27Ala > Gly24Ala > Gly24Ala/Gly27Ala (most resistant).

DISCUSSION

The approach that we are taking to the problem of tropomyosin function at low temperature is to utilize sources of the protein that in the natural setting exist as a single isoform[39] and present a manageable number of substitutions. For example, the muscle in Atlantic salmon that powers high-speed swimming[40] is entirely composed of an α-type tropomyosin that shares just 20 substitutions with the more heat-resistant mammalian homologue.[39,41] In addition to the noncanonical amino acids that exist in the latter, including Gly 126, Asp 137, Tyr 214, Glu 218, Tyr 221, Gln 263, and Tyr 267,[2,26,27,42,43] the protein from salmon has glycines at positions 24 and 27 and an extra core hydroxyl group, threonine 179(d). Restoring alanine (the residue in rabbit α-tropomyosin[2]) at position 179 brings about a significant increase in melting temperature (Figure 1 and Table 1) as well as greater resistance to proteolytic digestion at Leu 169, 10 residues away (Figure 2A). Threonine is therefore thought to be destabilizing at this site relative to alanine, consistent with the reported effects of these two amino acids at the "d" position of a synthetic coiled-coil peptide.[44]

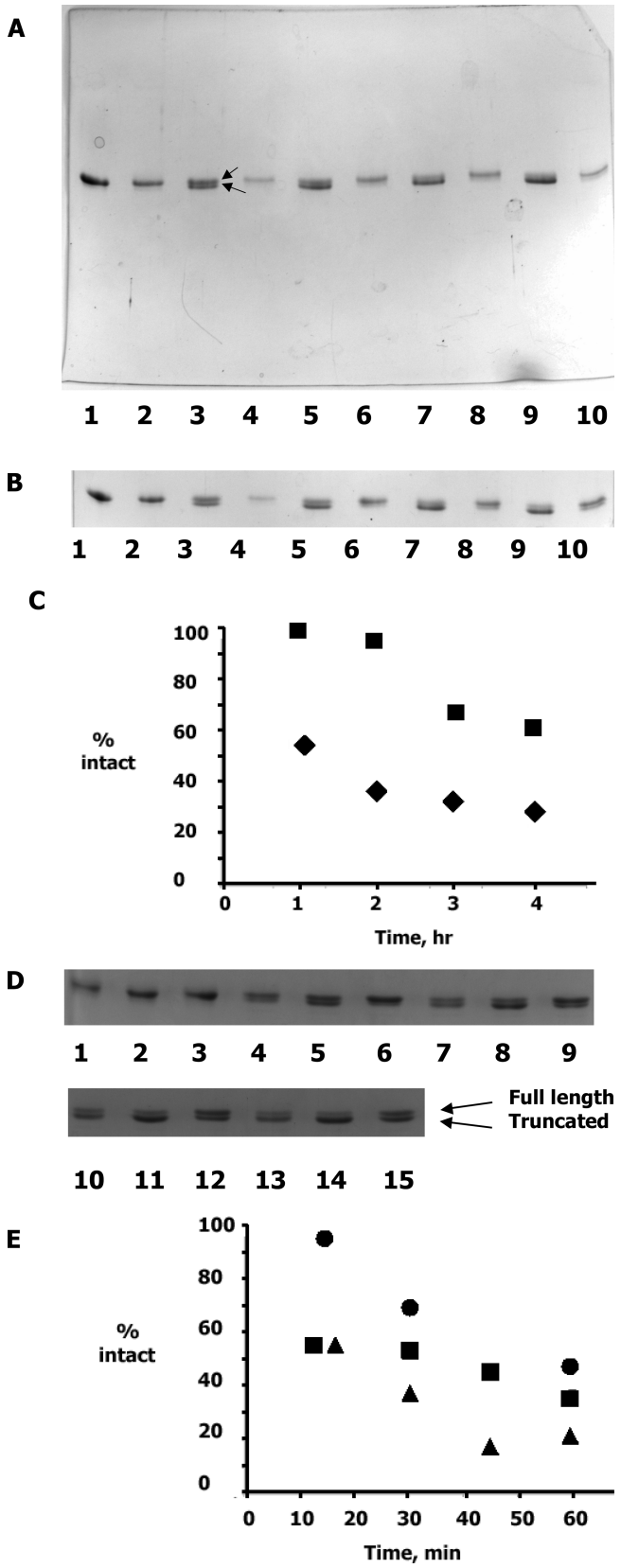

Figure 3. Comparison of the susceptibility of recombinant (nonmutant and glycine to alanine mutants) Atlantic salmon fast skeletal tropomyosin to digestion by outer membrane protease T (Omp-T). Assay buffer consisted of 0.1 M NaCl, 50 mM sodium phosphate, 5 mM EDTA, and 1 mM DTT (pH 7.0). The temperature was 10 °C. Comparison was conducted side by side (i.e., at the same time).

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Figure 3. continued

Reaction was initiated by addition of tropomyosin and continued while the sample was constantly mixed. To terminate the reaction, aliquots (mutant and control) were centrifuged to pellet the cells. The supernatant was then immediately boiled in the presence of SDS. Approximately 1 μg of protein was loaded on a 12% (w/v) SDS−polyacrylamide gel that was stained with Coomassie Brilliant Blue R-250. The reason for the diffuse staining apparent in lane 4 of panels A and B is unknown. (A) SDS−PAGE analysis of the time course of Omp-T cleavage of Gly24Ala mutant and control (nonmutant) tropomyosins at 10 °C. The full (nonsectioned) Coomassie-stained gel is presented to confirm that proteolysis had not occurred at more than one site. Top arrow (lane 3), intact tropomyosin. Bottom arrow, truncated (residues 7−284) tropomyosin.[35] Control: lanes 1, 3, 5, 7 and 9. Gly24Ala mutant: lanes 2, 4, 6, 8, and 10. Digestion times: no enzyme (lanes 1 and 2), 60 min (lanes 3 and 4), 120 min (lanes 5 and 6), 180 min (lanes 7 and 8), and 240 min (lanes 9 and 10). (B) Time course of Omp-T cleavage of Gly27Ala mutant and control (nonmutant) tropomyosins at 10 °C. Conditions were identical to those described for panel A. Only the section of the stained gel containing intact and amino-terminally truncated tropomyosin is shown. Control: lanes 1, 3, 5, 7 and 9. Gly27Ala mutant: lanes 2, 4, 6, 8, and 10. Digestion times: no enzyme (lanes 1 and 2), 60 min (lanes 3 and 4), 120 min (lanes 5 and 6), 180 min (lanes 7 and 8), and 240 min (lanes 9 and 10). (C) Densitometric analysis (using AlphaView version 3.40) of the gel image in panel B. The amount of intact tropomyosin (top band) as a percentage of the total (i.e., intact + truncated) is plotted vs time: (◆) control (nonmutant) and (■) Gly27Ala. The authors acknowledge that the band percentages as determined by this method are approximate. (D) SDS−PAGE analysis of the time course of Omp-T cleavage of single and double glycine to alanine mutant tropomyosins. The three mutants were digested side by side at 25 °C. As in panel B, only a section of the Coomassie Brilliant Blue stained gel is shown. Gly24Ala mutant: lanes 1, 4, 7, 10 and 13. Gly27Ala mutant: lanes 2, 5, 8, 11 and 14. Gly24Ala/Gly27Ala mutant: lanes 3, 6, 9, 12, and 15. Digestion times: no enzyme (lanes 1−3), 15 min (lanes 4−6), 30 min (lanes 7−9), 45 min (lanes 10−12), and 60 min (lanes 13−15). The experiment was repeated on three separate occasions with identical results. (E) Densitometric analysis (using AlphaView version 3.40) of the gel images in panel D. The amount of intact tropomyosin (top band) as a percentage of the total (i.e., intact + truncated) is plotted vs time: (●) Gly24Ala/Gly27Ala, (■) Gly24Ala, and (▲) Gly27Ala. Again, we acknowledge that the band percentages as determined by this method are approximate.

While heterogeneity within the core of tropomyosin is a theme that dates back to the 1970s, the existence of neighboring glycines is new. Given their amino-terminal location, it is not overly surprising that mutagenesis leads to a change in thermal melting smaller than that of the aforementioned centrally located mutation. Mutating one glycine was inconsequential in this regard (Figure 1 and Table 1). Mutating both of them increases the melting temperature by 1 °C relative to the control (Figure 1 and Table 1), similar in magnitude to that reported for Gly 126.[26] Conversely, marked differences in the protease sensitivity of the amino-terminal region are demonstrated. Swapping glycine for alanine confers resistance to Omp-T-directed cleavage (Figure 3A−E) at the sixth peptide bond, Lys 6−Lys 7.[35] By inference, the greater sensitivity of the control can be attributed to the glycyl pair (which may, for example, alter the physical accessibility of the cleavage site). Each glycine is important in this regard. The double mutant displays resistance to Omp-T greater than those of the two single mutants that are not equivalent to each other (Figure 3D,E), something that may reflect the proximity of a given glycine to the scissile bond. It should be noted that the digestion experiments in panels A and B of Figure 3 were performed at 10 °C, not far removed from that of the natural habitat.

Acetylation of Met 1 is known to affect several properties of tropomyosin[45−51] and is relevant here because the recombinant protein was synthesized in a prokaryotic host. Previously, this laboratory reported that acetylated salmon tropomyosin requires an incubation with Omp-T to achieve 100% truncation that is longer than that of the unacetylated form (Figure 1F of ref 35). Further, the same trend was demonstrated for the wild-type tropomyosins from rabbit and salmon,[35] something that we now attribute to the glycyl pair. Hence, both amino-terminal acetylation and the special pair of glycines are thought to be determinants of local stability.

The locations of the isomorphisms under investigation coincide with known interactive regions within tropomyosin. The fact that mutation of Thr 179 affects Ca(II) activation of actomyosin MgATPase (Table 1) is consistent with this residue being located near, or within, a region involved in binding to troponin[33,52] or actin.[53] Threonine 179 is present in a number of fishes, including species that are endothermal such as shark[41] and tuna.[54] (While endothermy provides several advantages, the temperature of the trunk is typically well below 37 °C.) By comparison, Gly 24 and Gly 27 are rare. While the initial functional analysis was inconclusive (Table 1), the influence of the glycyl pair must extend to tropomyosin's overlap region because the sole Omp-T cleavage site is at Lys 6.[35] Alternatively, or in addition, they may be required for ion pairing between Lys 7 in tropomyosin and Asp 25 in actin.[55−57] Intriguingly, cardiac tropomyosin in trout (a close relative of salmon) also possesses a double glycine but at positions 83 and 87,[28] near the alanine cluster required for F-actin binding.[15]

In conclusion, an α-tropomyosin from Atlantic salmon, a cold-blooded marine vertebrate, is demonstrated to contain strategically positioned destabilizing amino acids that mesophilic homologues do not have, closely placed glycines being a case in point. The isomorphisms under investigation are suggested, in all likelihood, to generate additional molecular flexibility by which to oppose the threat of cold-induced rigidity.

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