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The Carboxyl Terminal Amino Acid Residues Glutamine276-Threonine277 Are Important for Actin Affinity of the Unacetylated Smooth α-Tropomyosin

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Tropomyosin (TM) is an important actin binding protein involved in regulation of muscle contraction. Unacetylated striated tropomyosin failed to bind to actin whereas unacetylated smooth tropomyosin bound well to actin. It has been demonstrated that high actin affinity of unacetylated α-tropomyosin was ascribed to the carboxyl terminal amino acid residues. In order to define the role of the carboxyl terminal residues of tropomyosin molecule on actin binding, two mutant tropomyosins were constructed. TM11 is identical to the striated tropomyosin except that the carboxyl terminal last three amino acids was replaced with ^{282}NNM^{284} whereas in TM14 ^{276}HA^{277} was substituted with smooth specific ^{276}QT^{277}. TM11 and TM14 were overproduced in *Escherichia coli* and analyzed for actin affinity. The apparent binding constants (Kapp) of unacetylated tropomyosins were $2.2\times10^6M^{-1}$ for sm9, $1.03\times10^6M^{-1}$ for TM14, $0.19\times10^6M^{-1}$ for TM11, $>0.1\times10^6M^{-1}$ for striated, respectively. This result indicated that higher actin affinity of the unacetylated smooth tropomyosin was primarily attributed to the presence of QT residues in the smooth sequence. In case of the Ala-Ser (AS) dipeptide extension of the amino terminus of tropomyosin, Kapp were $21.1\times10^6M^{-1}$ for AS-sm9, $8.0\times10^6M^{-1}$ for AS-11, $4.7\times10^6M^{-1}$ for AS-14, $3.8\times10^6M^{-1}$ for AS-striated. AS-TM11 showed considerably higher actin affinity than AS-TM14, implying that interaction of Ala-Ser of the amino terminus with the carboxyl terminal residues. Since Kapp of AS-TM11 was significantly lower than that of AS-sm9, the presence of QT might be required for restoration of high actin affinity of the smooth α-tropomyosin. These results suggested that the carboxyl terminal amino acid residues Glutamine275-Threonine276 are important for actin affinity of the recombinant smooth α-tropomyosin, particularly of unacetylated smooth α-tropomyosin.

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Introduction

Tropomyosins (TM) are a family of highly conserved coiled-coil actin binding proteins present in virtually all of eukaryotic cells. Muscle tropomyosins are fibrous molecules composed of two polypeptide chains of 33,000 Da (284 amino acid residues) each in a two-stranded coiled-coil configuration. It is localized in the long pitch grooves of actin filament and stabilizes and stiffens actin filament. The function of tropomyosin in striated muscle is in association with the troponin complex to regulate interaction of actin and myosin in a calcium sensitive manner (for reviews, Zot and Potter, 1987; Tobacman, 1996). In smooth muscle and nonmuscle cells where troponin is absent, the role of tropomyosin is less understood but it has been generally agreed that tropomyosin is involved in thin filament regulation with caldesmon. In nonmuscle cells, tropomyosin may function as a modulator of microfilaments (Pittenger *et al.*, 1994 for review).

Although tropomyosins are present in many types of cells, different forms of the proteins are characteristics of specific cell types and these tissue specific isoforms of tropomyosin show functional differences. At least 15 different isoforms were generated from the use of alternative promoters and alternative splicing of the transcripts of a small number of genes. As a consequence of alternative splicing, striated and smooth muscle α-tropomyosins differ only in exon2 (amino acid residues 38-80) and exon 9 (amino acid residue 258-284) regions.

Functions common to all TMs are to bind actin cooperatively to F-actin (Yang *et al.*, 1979; Zot and Potter, 1988 for review). Since muscle tropomyosin spans the length of 7 actin monomers and sequence analysis suggested the presence of 7 repeats each containing 42 amino acids, it was postulated that tropomyosin contains 7 actin binding sites (McLachlan and Stewart, 1976). Although the periodic nature

of actin binding sites is well established, both ends of the molecule may be more important for actin binding than the postulated sites. It has been indicated that local changes at the amino terminus of tropomyosin greatly influence the actin affinity (Heald and Hitchcock-DeGregori, 1988; Cho *et al.*, 1990).

Recombinant unacetylated smooth TM bound strongly to actin while unacetylated striated TM bound poorly in the absence of troponin (Cho and Hitchcock-DeGregori, 1991). Since both chimera sm9, which has the striated exon 2 and smooth exon9 and smooth TM bound well to actin and the actin binding affinities of unacetylated smooth tropomyosin and sm9 were comparable to each other, the higher actin affinity of recombinant smooth TM was attributed to the smooth specific exon 9 (amino acid residue 258-284), which is located in the carboxyl terminus. Hammell and Hitchcock-DeGregori constructed a chimera TM9a/d which was identical to the striated except that the carboxyl terminal last 9 amino acid residues were changed to smooth specific sequences. They showed that sm9 and TM9a/d were indistinguishable to each other in actin binding affinity so that it was concluded that the higher actin affinity was due to the presence of the last 9 carboxyl terminal amino acid residues (Hammell and Hitchcock-DeGregori, 1996).

In present work two mutant recombinant tropomyosins, TM11 and TM14 were constructed to define the role of the carboxyl terminal amino acid residues on actin binding affinity. Recombinant TM11 and TM14 were purified and analyzed for actin binding. The results showed that the carboxyl terminal amino acids Glutamine275-Threonine276 are important for actin affinity of unacetylated smooth α-tropomyosin.

Materials and Methods

Construction and expression of mutant recombinant tropomyosins Rat striated α-tropomyosin cDNA was a gift from Dr. B. Nadal-Ginard of Harvard Medical School (Ruiz-Opazo and Nadal-Ginard, 1987). The cDNA was 1.2 kb long and was cloned into EcoR1 site of pUC119 and resulting plasmid was termed pUC119/ST. To construct the mutant tropomyosins a double polymerase chain reaction (megaprimer) method (Sarkar and Sommer, 1990) was employed using *Pwo* DNA polymerase (Boeringer-Manheim, Germany). The template DNA, pUC119/ST, was at first amplified in a Perkin Elmer thermal cycler Model 480 with 5' mutagenic primers (for TM11, CAACGATATG **AACAACATG**TAAGTTTCT, 28mer, the underlined and the bold face were AflIII restriction site introduced and codons for altered amino acids, respectively; for TM14, CAGCGAGGAGCTG GAGCTGGA**CCAGACTTT**AAACGATATGAT, 37mer, DraI restriction site) and with 3' primer (M13/pUC reverse sequencing primer; 19mer). After denaturation of the template DNA at 94°C for 90 sec, the first PCR reaction was carried out by 25 cycles of 92°C for 90 sec, 55°C for 1 min, and 72°C followed by final 6 min incubation at 72°C. The amplified PCR product, approximately 350 bp long, was used as a primer for second PCR. The second PCR was performed using pUC119/ST as a template with 5'-primer (M13/pUC sequencing primer; 32mer) and with 3'-megaprimer obtained from the first PCR. The amplification conditions were as follows; 94°C for 2 min denaturation, 30 cycles, 94°C for 90 sec, 64°C for 90 sec, 72°C for 2 min, and final 7 min incubation at 72°C. The amplified PCR products were digested with restriction enzyme NcoI and BamHI. The resulting fragments were ligated into the NcoI-BamHI sites of the expression vector pET11d (Novagen, Madison, USA) and transformed to DH5α. The plasmids were isolated and were screened for mutants with appropriate restriction enzymes. Ala-Ser dipeptide fusion tropomyosins were constructed with a 5' mutagenic primer whose sequence was CCACCGCCACC**ATG GCTAGC**ATGGACGCCA TCAAG (35mer; the underlined is NheI restriction site) and a 3'-primer (primer D; 31mer, T7 terminator primer) utilizing pET11d/ST, pET11d/sm9, pET11d/TM11, and pET11d/TM14 as templates. PCR conditions were identical to those of the second PCR described above. The entire coding regions of pET11d/TM11 and pET11d/TM14 were autosequenced to confirm that no amino acid sequences were changed accidentally during PCR. The pET11d plasmids producing unacetylated and Ala-Ser dipeptide tropomyosins were transformed into *E. coli* strain BL21 {DE3} and were overexpressed for 4h by an addition of 0.3 mM of isopropyl-β-thiogalactopyranoside (IPTG). Routine procedures for preparing and handling of recombinant DNA and culture of bacteria were conducted as described in methods manuals (Sambrook *et al.*, 1989) or as suggested by the manufacturers or the suppliers. All restriction enzymes and DNA modifying enzymes were purchased from Boeringer-Mannheim (Mannheim, Germany). Oligonucleotide primers were synthesized by and were purchased from Bioneer (Taejon, Korea). Authosequencing was conducted at KAIST Bio Medical Reserch Center in Taejon, Korea.

Isolation and purification of recombinant tropomyosins Recombinant tropomyosins were purified as reported previously (Cho *et al.*, 1990) with modifications. In brief, bacterial cells were harvested after induction with IPTG and lysed by lysozyme and freeze-thaw treatments. After sonication total lyzate was centrifuged for 20 min at 18,000 rpm in a Sorvall SS34 rotor. 5 M NaCl was added to the supernatant to a final concentration of 1 M and placed in a boiling water bath for 5 min and stood for 1 h at room temperature to cool slowly. The heat-denatured proteins were removed by centrifugation for 20 min at 15,000 rpm in a Sorvall SS34 rotor. The supernatant was precipitated with 35 to 70 percent ammonium sulfate and the pellet was dissolved in and was dialyzed against 20 mM Tris-HCl, pH 7.5, and 0.5 mM DTT. The dialyzed proteins were purified by DE52 DEAE-cellulose ion exchange column chromatography. The column was equilibrated with 20 mM Tris-HCl, pH 7.5, and 0.5 mM DTT and eluted with a linear gradient of 0 to 0.6 M NaCl containing the same buffer. The fractions containing tropomyosin were pooled and purified further by hydroxyapatite column chromatography as described previously (Cho *et al.*, 1990). Chicken pectoral muscle actin was extracted from acetone powder and was purified as described (Hitchcock-DeGregori *et al.*, 1982). Protein concentration was determined by Bradford method using bovine serum albumin as a standard (Bradford, 1976). All chemicals were reagent grade and

were purchased from Sigma Chemical Co (St. Louis, USA) unless specific suppliers are indicated in the text.

Actin binding assay The actin binding assay for tropomyosin was carried out by cosedimentation of proteins at room temperature as described (Cho *et al.*, 1990). The conditions of the actin binding assays are described in the figure legends. Actin and tropomyosin were combined in an Eppendorf microcentrifuge tube and vortex thoroughly. The mixture, total volume of 200 µl, was centrifuged at 50,000 rpm for 40 min at 25°C in Beckman TLA rotor using a Beckman Table Top Ultracentrifuge (Model TL-100). Following centrifugation the supernatant was transferred, and the pellet was rinsed with and was resuspended in 35 µl of the binding assay buffer. The pellets and supernatants were run on 12% SDS-polyacrylamide gel electrophoresis and the gels were stained with Coomassie Blue. Tropomyosin bands of the supernatants and tropomyosin and actin bands of the pellet were quantified by using a BioRad scanning densitometer Model GS-700. Binding constant (Kapp) and Hill coefficient (α^H) were obtained using SigmaPlot 2000 (SPSS) by fitting the data to the following equation.

$$v = n[TM]^{\alpha H} \cdot Kapp^{\alpha H} / 1 + [TM]^{\alpha H} \cdot Kapp^{\alpha H}$$

The data were normalized because the intensity of the staining was somewhat variable from experiment to experiment.

Results and Discussion

Previous results revealed that the carboxyl terminal last 9 amino acids define the higher actin affinity of unacetylated smooth α-tropomyosin (Hammell and Hitchcock-DeGregori, 1996). In order to determine the carboxyl terminal responsible for higher actin affinity, two mutant tropomyosins, TM11 and TM14, were constructed as a first step to assess the role of each of the residue. Among 9 amino acids (residue 275-284) Leu278 is identical and residues 280 (Glu for striated, Asp for smooth) and 281 (Met for striated; Leu for sm9) are conserved changes as shown in Table 1. TM11 is identical to the striated tropomyosin except that ^{276}HA^{277} (His-Ala) residues were altered to ^{276}QT^{277} (Gln-Thr); TM14 has smooth specific ^{282}NNM^{284} in lieu of ^{282}TSI^{284} of the striated. The mutant TMs were constructed by site-directed mutagenesis and were overexpressed in *E. coli* as well as striated and sm9 for comparative studies on actin binding. The mutant TMs remained soluble upon lysis and were heat-stable. After hydroxyapatite column chromatography step all recombinant tropomyosins were purified near to homogeneity, 95-99 percent homogeneity as determined by densitometry. Minute impurities of low molecular weight were thought be breakdown fragments of tropomyosin. Subsequent actin binding assay indicated that the minor band did not bind to actin and showed no interference with actin binding assay (data not shown).

Binding of unacetylated tropomyosin to actin The actin binding assay was performed with purified recombinant unacetylated tropomyosin. The assay was carried out by cosedimentation of proteins. As shown in Figure 1, unacetylated sm9 and TM14 bound well to actin while TM11 bound poorly to actin and striated TM hardly bound to actin. Since striated TM and TM14 differ only in residues 275 and 276, this result indicated that high actin affinity of sm9 was attributed to the presence of ^{275}QT^{276} residues of the smooth exon9.

To assess the role of the amino acid residues on actin

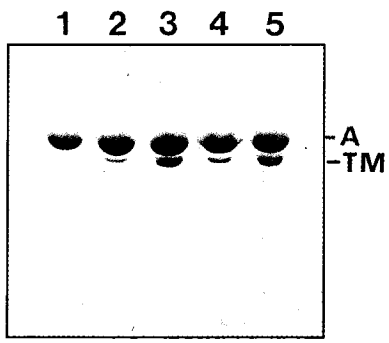

Fig. 1. SDS-polyacrylamide gel analysis of pellets of actin binding assay with unacetylated tropomyosins. Conditions: 5 µM actin, 2 µM unacetylated tropomyosin, in 10 mM imidazole, pH 7.0, 150 mM NaCl, 2 mM $MgCl_2$, 0.5 mM DTT. The proteins were cosedimentated as described under "Materials and Methods". The pellets were analyzed by SDS-polyacrylamide gel electrophoresis on 12% gel. Lanes: 1, actin (**A**) alone; 2, actin, striated tropomyosin (**TM**); 3, actin, sm9 TM; 4, actin, TM11; 5, actin, TM14

Table 1. The carboxyl terminal 9 amino acids sequences of striated, smooth (sm9), TM11 and TM14

	276	277	278	279	280	281	282	283	284
striated (ST)	H	A	L	N	D	M	T	S	I
smooth (sm9)	Q	T	L	L	E	L	N	N	M
TM11	H	A	L	N	D	M	N	N	M
TM14	Q	T	L	N	D	M	T	S	I
heptad repeat	*c*	*d*	*e*	*f*	*g*	*a*	*b*	*c*	*d*

TM11 was identical to striated sequence except that the last three residues were substituted to NNM of sm9. TM14 has QT of sm9 instead of HA and the rest of the sequence is same as striated. The lower case letters *a* and *d* correspond to the interface residues in the heptapeptide (heptad) repeat of coiled coil structure.

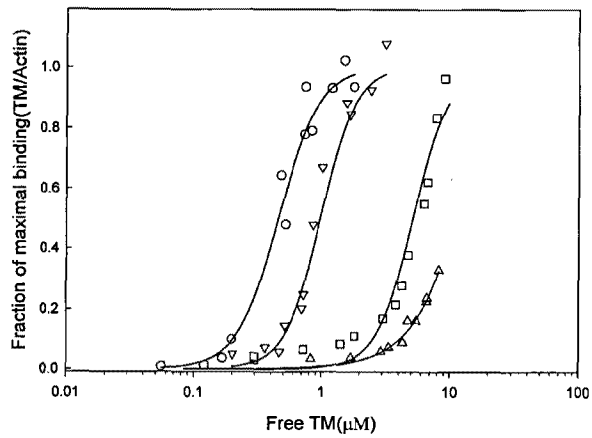

Fig. 2. Binding of unacetylated tropomyosins to actin. Conditions: 5 μM actin, 0-10 μM tropomyosin, in 10 mM imidazole, pH 7.0, 150 mM NaCl, 2 mM $MgCl_2$, 0.5 mM DTT Unacetylated tropomyosins and actin were cosedimentated as described under "Material and Methods". The supernatants and pellets were analyzed by SDS-polyacrylamide gel. TMs in pellets (bound) and the superantants (free) were quantified by densitometry and the data were fitted to the Hill equation using a SigmaPlot2000. Symbols: ○, sm9; □, TM11; ▽, TM14; △, striated.

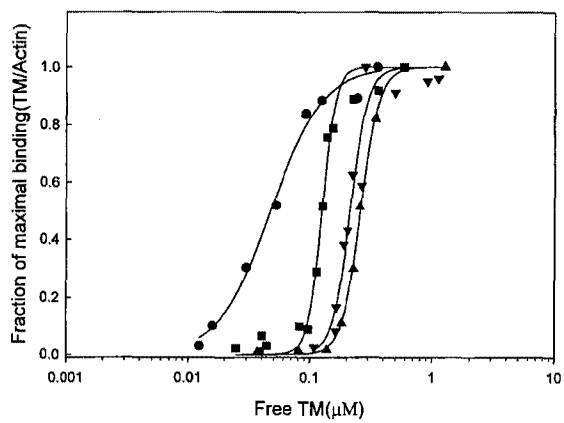

Fig. 3. Binding of Ala-Ser dipeptide fusion tropomyosins (AS-TMs) to actin. Conditions: 5 μM actin, 0-3 μM tropomyosin, in 10 mM imidazole, pH 7.0, 150 mM NaCl, 2 mM $MgCl_2$, 0.5 mM DTT. Ala-Ser dipeptide fusion (AS)-TM and actin were cosedimentated as described under "Materials and Methods". Symbols: ●, AS-sm9; ■, AS-TM11; ▼, AS-TM14; ▲, AS-striated.

affinity further, the assay for binding isotherm of tropomyosin to actin was conducted to determine the strength of actin affinity quantitatively. The results were shown in figure 2 and in Table II. The apparent binding constants (Kapp) and Hill coefficients (α^H) were obtained from the data by the curve-fitting with SigmaPlot. Kapp were $2.2 \times 10^6\ M^{-1}$ for sm9, $0.19 \times 10^6\ M^{-1}$ for TM11, $1.03 \times 10^6\ M^{-1}$ for TM14, respectively. Kapp for striated TM was difficult to obtain under the conditions that the actin binding assay was conducted even though it was estimated to be approximately $0.03 \times 10^6\ M^{-1}$ by the curve-fitting. Since actin affinity of the TM14 was significantly higher, more than 5 fold, than that of the TM11, this result supported the finding that ^{276}QT^{277} residues are primarily responsible for high actin affinity of smooth unacetylated TM. It has been reported that removal of the last three residues of tropomyosin has little effect on actin binding (Ueno *et al*, 1976; Johnson and Smillie, 1977) whereas carboxypeptidase A-digested tropomyosin, which lacks 11 carboxyl-terminal amino acids failed to bind to actin in the absence of troponin (Mak and Smillie, 1981). Thus residue 275 and 276 are more important for actin binding rather than the carboxyl terminal last three amino acids. Unacetylated TM11, however, bound stronger to actin than striated TM so that the carboxyl terminal ^{282}NNM^{284} was also required for high actin affinity.

Tropomyosin has a two-chained α-helical coiled coil structure. The characteristics of the coiled coil was heptapeptide or heptad repeat alternating nonpolar and polar amino acids, normally nonpolar residues such as Leu at *a* and *d* positions. Structure of the extreme ends of tropomyosin molecule was unknown and the ends of tropomyosin may not be α-helical (McLachlan and Stewart, 1975; Phillips, *et al.*, 1986; Whitby, *et al.*, 1992). Nonetheless heptad repeat remained intact to the carboxyl terminal last amino acid (Table 1). The residues QT/HA correspond to *c* and *d* position in heptad repeat. Higher actin affinity of QT may be interpreted that these residues were directly involved in an interaction between tropomyosin and actin molecules. Alternatively these residues confer an increased stability to tropomyosin molecule. The latter seemed less likely to be an explanation for higher actin affinity. The change from Ala to

Table 2. Actin binding constants and Hill coefficients of recombinant tropomyosins

	Unacetylated		Ala-Ser dipeptide fusion	
	K_{app} (10^6 M)	α^H	K_{app}(10^6 M)	α^H
striated	<0.1	N.D.	3.8±0.04	5.7±0.10
sm9	2.2±0.19	2.7±0.61	21.1±1.7	2.0±0.27
TM11	0.19±0.03	3.1±0.50	8.0±0.16	6.0±1.4
TM14	1.03±0.07	2.2±0.19	4.7±2.0	7.8±1.2

The data from the binding isotherms shown in Figure 2 and 3 were fitted to the Hill equation. Data were reported as *K*app (apparent binding constant) and α^H (Hill coefficient)±Standard Error calculated using SigmaPlot2000 (SPSS).

Thr at *d* position tends to make tropomyosin molecule locally less stable since the coiled coil structure of tropomyosin was mainly stabilized by hydrophobic interaction between the interface nonpolar amino acid residues at *a* and *d* positions (Greenfield and Hitchcock-DeGregori, 1995).

Binding of Ala-Ser dipeptide fusion tropomyosin to actin Unlike muscle tropomyosin that the amino terminus is blocked with acetyl group, bacterially produced recombinant tropomyosins were unacetylated due to the lack or shortage of N-acetylation machinery. Unacetylated tropomyosin bound poorly to actin, was nonpolymerizable, and failed to regulate ATPase (Cho *et al.*, 1990). The addition of two amino acids, Ala-Ser, to the amino terminus of tropomyosin was sufficient for the function of the N-acetyl group of muscle tropomyosin. The Ala-Ser dipeptide fusion tropomyosin exhibited similar circular dichroism spectra and thermal stability to muscle tropomyosin and restored the functional properties present in the muscle tropomyosin such as actin binding, head-to-tail polymerization, capacity to inhibit acto-myosin ATPase, and induction of cooperative equilibrium binding of myosin subfragment 1 to actin (Monteiro *et al.*, 1994; Maytum *et al.*, 2000).

As shown in Figure 3 and summarized in Table 2, all Ala-Ser dipeptide (AS)-TMs markedly increased actin affinities as compared to the unacetylated tropomyosin. Kapp were 21.1×10^6 M^{-1} for AS-sm9, 8.0×10^6 M^{-1} for AS-TM11, 4.7×10^6 M^{-1} for TM14, 3.8×10^6 M^{-1} for AS-striated, respectively. Among them AS-striated was increased most and AS-TM14 was least. This may suggest that an interaction of Ala-Ser residues on the amino terminus of a neighboring tropomyosin molecule with the carboxyl terminal residues resulted in higher actin affinity. Implication of the interaction between residues of the carboxyl and amino termini is consistent with a previous report that the Ala-Ser dipeptide extension tropomyosin was polymerizable, indicative of head-to-tail interaction (Monteiro *et al.*, 1994).

In contrast to the unacetylated tropomyosins, actin affinity of AS-TM11 was significantly higher than that of AS-TM14. Apparently the carboxyl terminal last three amino acid residues are important for higher actin affinity in case of AS-TM. It may be possible that the effect of QT on higher actin affinity be diminished by the greater effect of the last three amino acids resulted from the interaction between two termini. Nevertheless, the actin affinity of AS-TM 11 was significantly lower than that of AS-sm9 and actin affinity of AS-14 was to some extent higher than that of AS-striated. Thus it was suggested that QT was still required for higher actin affinity although AS-TM11 bound stronger than AS-TM14.

It has been reported that tropomyosin molecules polymerize head to tail fashion as observed by increase in viscosity and it had been postulated that the cooperative actin binding is governed by the strength of the head-to-tail interaction (McLachlan and Stewart, 1975). Polymerization is not prerequisite for high actin affinity and the strength of the head-to-tail interaction between tropomyosin molecules showed no direct correlation with the cooperativity in actin binding. It has been known that several nonpolymerizable tropomyosins bound to actin strongly in the absence of troponin (Heald and Hitchcock-DeGregori, 1988; Cho *et al*, 1990; Cho and Hitchcock-DeGregori, 1991; Sano *et al.*, 2000). Except AS-sm9 all three AS-TMs showed increase more than 2 fold in Hill coefficient, a measure of cooperativity. The Hill coefficients of all three AS-TMs were comparable to each other ranging from 5.7 to 7.8. Interestingly Hill coefficient of AS-sm9 was 2.0 and was slightly decreased in spite of 10-fold increase in actin affinity as compared to unacetylated sm9.

Judging from the results from present work, the role of the carboxyl terminal residues on actin affinity should be considered in association with the amino terminal modification. Unacetylated tropomyosin evidently showed the importance of QT residues in actin affinity but the amino terminal modification also played a significant role in actin affinity as demonstrated with AS-TMs. Consequently, these results from this work supported previous findings that both ends, the amino terminus and the carboxyl terminus of tropomyosin molecule are crucial for actin binding.

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