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Functions of α-Tropomyosin Are Mainly Dependent upon the Local Structures of the Amino Terminus

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It has been previously reported that unacetylated α-tropomyosin(TM) produced in *E. coli* failed to bind to actin while acetylated muscle TM and Ala-Ser dipeptide fusion TM (AS-TM) bound well to actin. In order to determine the structural requirement of the amino terminus for high actin affinity, a recombinant tropomyosin (Ala-TM) that a single Ala residue was added to the amino terminus of α -TM was constructed, overexpressed, and purified from *E. coli*. Actin affinity of Ala-TM was $2.3x10^6M^{-1}$, whereas that of unacetylated TM was considerably lower than $0.1x10^6M^{-1}$ indicating that addition of a single Ala residue to the amino terminus drastically increased, at least twenty times, actin affinity of TM. Ala-TM, however, bound to actin about three times weaker than acetylated TM and AS-TM, implying that the addition of an Ala residue was insufficient for complete restoration of high actin affinity. While Ala-TM, AS-TM, and muscle TM showed inhibition and activation of actomyosin S1 ATPase activity depending on myosin S1 concentration, the degree of inhibition and activation was different from each other. AS-TM exhibited the greatest inhibition of the ATPase at low S1 concentration, whereas the greatest activation of the ATPase was observed with muscle TM. These results, together with previous findings, strongly suggested that local structure of the amino terminus is the crucial functional determinant of TM.

Key words – recombinant tropomyosins, actin binding, myosin S1 ATPase, N-acetylation

Tropomyosin (TM), which is an important regulatory protein in contractile system, is a family of proteins that are highly conserved and present in all types of muscle tissue and in many of nonmuscle cells [14,35]. It has high α-helix content (over 90%) and normally present in dimer. Muscle tropomyosins are fibrous molecules composed of two polypeptide chains of 33,000 (284 amino acid residues) each in a two-stranded coiled coil configuration. Two polypeptide chains are aligned in parallel and in register. The function of tropomyosin in skeletal and cardiac muscle is in association with the troponin complex to regulate interaction of actin and myosin in a calcium sensitive manner.

Function common to all TMs is to bind cooperatively to F-actin. Muscle tropomyosin spans the length of 7 actin monomers. Although periodic nature of actin binding site is well established, both ends of the molecule, that is the amino (N) and carboxyl (C) terminal regions may be more important for actin binding than periodic actin binding sites. It has been indicated that local changes at the amino terminus greatly influence the actin affinity [5,10]. It has been also reported that the carboxyl terminal 9 amino acid residues define actin affinity of tropomyosin [3,9].

Some 60-90% of eukaryotic proteins synthesized in the cytoplasm are isolated with their amino terminal acetylated [25]. Acetylation is common only for proteins made in cytosol, but not in mitochondria or chloroplast in eukaryotic cells. A variety of Nα-acetyltransferase enzymes are thought to catalyze this reaction, using acetyl-CoA as the acetyl donor. Acetylation can occur whether or not the initiating Met residue is still present. Whether acetylation occurs depends to some extent on the nature of the N-terminal residue. In a survey using mutagenesis of the N-terminus of one particular protein, those forms acetylated had N-terminal Gly, Ala, Ser, and Thr residues. The initiating Met was retained and acetylated if the following residue was Asp, Glu, or Asn. Nevertheless, many exceptions to these rules are found in various proteins, and so it must be other properties of the protein that determine whether or not it is acetylated.

Vast majority of muscle proteins including tropomyosin are acetylated. Unlike muscle tropomyosin, bacterially produced recombinant tropomyosin is unacetylated at the amino terminus due to the lack of N-acetylation enzyme machinery in *E. coli*. Unacetylated striated α-tropomyosin showed drastic changes in tropomyosin function. It bound

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poorly to actin failed to polymerize, and was unable to regulate myosin S1 ATPase activity in the absence of troponin [5]. Monteiro *et al.* [21] previously demonstrated that recombinant tropomyosin with Ala-Ser dipeptide extension at the amino terminus restored muscle tropomyosin functions.

In order to determine local structural requirement of the amino terminus of tropomyosin that is important for function, a mutant (Ala-TM) that a single alanine residue was added to the amino terminus was constructed by site-directed mutagenesis using polymerase chain reaction (PCR). After overexpression in *E. coli* and purification, actin binding ability and regulation of myosin S1 ATPase activity of Ala-TM was compared with acetylated muscle TM, AS-TM, and unacetylated TM to assess the effect of the amino terminus structure on tropomyosin functions.

Materials and Methods

Construction and Overexpression of Recombinant Mutant Tropomyosin

Oligonucleotide site-directed mutagenesis by polymerase chain reaction was employed for constructing a new recombinant tropomyosin that contains a single Ala residue on the amino terminus of tropomyosin. The mutagenic 5′ primer whose sequence was CCACCGCCACC**AT GGCT**ATGGACGCCATCAAG (32mer) and pUC/M13 reverse sequencing primer (32mer) as the 3′ primer were used for PCR. The plasmid pUC119/ST (rat striated α -tropomyosin cDNA, [28]) was used as a template. The conditions for PCR were as follows: the template was denatured at 94°C for 2 min and 64°C for 90 sec and 72°C for 90 sec with 25 cycles of amplification followed by 6 min at 72°C for final extension. The reaction was carried out in a Hybaid Gradient PCR Express using *Pwo* DNA polymerase.

The resulting PCR product was cleaned by Wizard PCR cleanup kit and digested with *Nco*I and *BamH*I. The *Nco*I-*BamH*I fragment of the PCR product was ligated into the *Nco*I-*BamH*I fragment of the expression vector pET11d and subsequently transformed into *E. coli* strain DH5α. The plasmid, designated pET11d/MA, was obtained and auto-sequenced the entire coding region of tropomyosin to confirm that mutagenesis occurred only at the position intended. Overexpression of the mutant tropomyosin was induced for 4 hr in *E. coli* strain BL21(DE3) by the addition of IPTG as described previously [3].

Protein Purifications

Recombinant tropomyosins were isolated and purified as described previously with minor modifications [3]. Briefly bacterial cells were harvested after induction and lysed with lysozyme treatment followed by sonication. Total lysate was centrifuged and NaCl was added to supernatant with a final concentration of 1 M NaCl. The supernatant was placed in a boiling water bath for 5 min and cooled gradually in a room temperature and then stood overnight in a cold room. The heat-denatured proteins were removed by centrifugation at 18,000 rpm for 20 min in a Sorvall SS34 rotor. Tropomyosins were precipitated with 35 to 70 percent of ammonium sulfate and the pellet was dissolved in and dialyzed overnight against DE52 DEAE-cellulose buffer (20 mM Tris buffer, pH 7.5, and 0.5 mM dithiothreitol(DTT)). The anion-exchange column was eluted with the same buffer containing a linear gradient of 0 to 0.6 M NaCl. Fractions containing tropomyosin were pooled and further purified by BioGel ceramic hydroxyapatite (BioRad) column chromatography. The column was equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing 1 M NaCl and 0.5 mM DTT and was eluted with a linear phosphate gradient from 50 mM to 250 mM sodium phosphate buffer, pH 7.0, containing 1 M NaCl and 0.5 mM DTT.

Chicken pectoral muscle actin was extracted from acetone powder, which was prepared from White Leghorn breast muscle and purified as described [13] and skeletal muscle tropomyosins was purified as described [14].

Myosin S1 (a generous gift from Dr. Winkelmann of UMDNJ Robert Wood Johnson Medical School) was prepared by digesting myosin from chicken pectoral muscle with papain as described [34]. Briefly, myosin was dialyzed against 0.2 M ammonium acetate, pH 7.2, 2 mM $MgCl_2$. The myosin suspension was digested with papain (final concentration of 10 μg/ml) for 8 min at 25°C and the reaction was stopped by the addition of iodoacetamide. The suspension was ultracentrifuged for 2 hrs at 50,000 rpm in a Sorvall TFT 50.38 rotor and the resulting supernatant was dialyzed against 50 mM imidazole, pH 7.0, 0.5 mM $MgCl_2$, 1 mM DTT. After dialysis the suspension was centrifuged for 20 min at 10,000 rpm in a Sorvall GSA rotor. The supernatant was then loaded onto a 2.5x2.5 cm DEAE-Sephacel column equilibrated with the dialysis buffer

and eluted with 800 ml of gradient from 0 to 0.5 M NaCl. Fractions of myosin S1 peak were pooled and lyophilized for storage.

Actin Binding Assay

Actin binding assay for tropomyosin was carried out by cosedimentation of F-actin and tropomyosins at room temperature in a Beckman Table Top Ultracentrifuge as previously described [5]. The conditions of the assays are described in the figure legends. The pellets and supernatants were run on 12% SDS-polyacrylamide gel electrophoresis [16]. Tropomyosin bands of the supernatant (free) and tropomyosin and actin bands of the pellet (bound) on the gels were quantified by densitometry. Binding constants were estimated using SigmaPlot 2000 by fitting the data to the Hill equation as described [3,15].

ATPase Assay

The ATPase activity was measured by colorimetrically determining inorganic phosphate released according to White [32]. Actomyosin S1 ATPase was measured as a function of myosin S1 or tropomyosin concentration. Myosin S1 was dialyzed extensively overnight against a buffer containing 5 mM imidazole (pH 7.0) 10 mM NaCl, 0.5 mM DTT to remove any residual phosphate. Actin stock was diluted to 32 μM with the same buffer for the dialysis of myosin S1 and tropomyosin was dialyzed against the same buffer prior to mixing.

The assay was carried out under conditions described in the figure legends. The reaction volume was 98 μl and the reaction time was 15-20 min depending on the type of experiment. The reaction was initiated by adding MgATP (typically a fifth of the reaction volume) and terminated by transferring 75 μl of the reaction mixture into a well of microtiter plate (96 well, 350 μl well volume, Nunc) containing 25 μl of the stop solution (13.3% sodium dodecyl sulfate in 0.12 M of EDTA in pH 7.0). Then 200 μl of the developing solution (0.5% (w/v) ferrous sulfate, 0.5% (w/v) ammonium molybdate in 0.5M sulfuric acid) was added and after standing for 20 min at room temperature, the microtiter plate was read at 655 nm using a BioRad Model 550 Microplate reader. A 20 mM potassium phosphate (KH_2PO_4) solution was used as a standard for the phosphate. Unless indicated, all the ATPase rates were corrected for the rate of hydrolysis by myosin S1 alone, 0.13-0.17 nmol of Pi/sec/nmol myosin S1, depending on the experiments.

All restriction enzymes and DNA modifying enzymes were purchased from Roche Molecular Biochemicals. All chemicals used were reagent grade from Sigma Chemicals Co. unless the specific suppliers were mentioned in the text. Oligonucleotide primers were purchased from Bionix and autosequencing was carried out at Macrogen using ABI Prism Model 3700.

Protein concentration was determined by either Bradford method [1] or measuring absorbance at 280 nm. The extinction coefficient (1% at 280 nm) used were 11.1 for actin, 8.3 for myosin S1, and 2.8 for tropomyosins, respectively. General recombinant DNA techniques were performed as described by Sambrook *et al.* [29] or as recommended by the manufacturers and supplies.

Results and Discussions

Preparation of Ala-TM

In order to address the local structural requirement for tropomyosin function a mutant was constructed using oligonucleotide-directed mutagenesis by polymerase chain reaction. The mutagenic primer encoded Met-Ala dipeptide fusion tropomyosin but the initiating Met residue is removed as overproduced in *E. coli*. Removal of the initiation methionine depends on the nature of the penultimate, that is, the second amino acid residue [12]. The initiating Met was followed by alanine and the resulting recombinant tropomyosin should have alanine residue at the amino terminus. Consequently the mutant recombinant tropomyosin was designated as Ala-TM

As shown in Fig. 1, Ala-TMs were soluble upon lysis and remained in stable and soluble form after heat treatment as the same characteristics shown in other recombinant TMs such as unacetylated TM and AS-TM [3]. Purification steps of mutant recombinant TMs included ammonium sulfate fractionation, DE52 DEAE-cellulose ion exchange, followed by hydroxyapatite column chromatography. All mutant TMs were successfully overproduced and purified to near homogeneity, over 95 percent as determined by scanning densitometry.

Actin Binding Function of TM

Tropomyosin is an actin binding protein and the actin binding is a fundamental function of tropomyosin. The

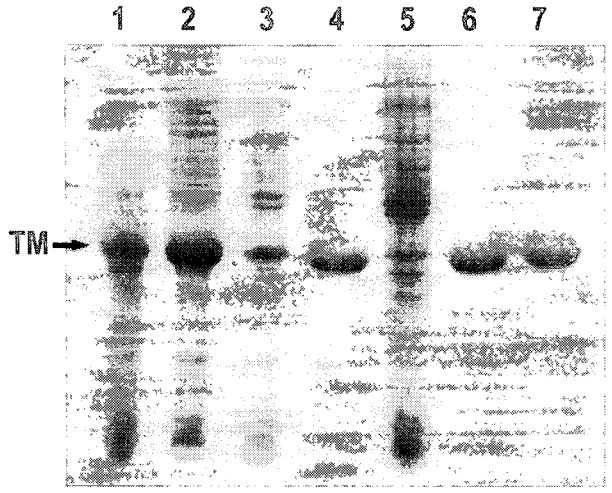

Fig. 1. SDS-polyacrylamide gel electrophoresis analysis of purification of mutant tropomyosin.
After induction for 4 hrs, each culture was harvested, lysed, and boiled for 5min. 5 μl of aliquots from each purification step were loaded onto 12% SDS-polyacrylamide gel.
Lane 1, total lysate; *lane 2*, supernatant after lysis; *lane 3*, pellet after lysis; *lane 4*, supernatant after boiling; *lane 5*, pellet after boiling; *lane 6*, pooled fractions after DE52 DEAE-cellulose chromatography; *lane 7*, pooled fractions after hydroxyapatite chromatography.

actin binding assay was performed by cosedimentaton with actin and tropomyosins at lower (1 μM) and higher (2 μM; saturating) concentrations of tropomyosin. As shown in Fig. 2, unacetylated TM hardly bound to actin while muscle(acetylated) TM and Ala-Ser-TM, bound well to actin as previously reported [3-5,10,21]. Ala-TM bound well to actin although Ala-TM appeared to bind slightly weaker than muscle and Ala-Ser TM. This result implied that a single Ala residue extension at the amino terminus was sufficient for restoration of actin affinity since Ala-TM bound much stronger than unacetylated TM.

To determine the actin affinity of TM quantitatively, the binding isotherm experiment was performed with recombinant tropomyosins. The result was shown in Fig. 3. The apparent binding constants of Ala-Ser TM, acetylated muscle TM, and Ala-TM were $6.8x10^6M^{-1}$ and $6.3x10^6M^{-1}$, and $2.3x10^6M^{-1}$, respectively. Due to the fact that unacetylated TM was bound poorly to actin and failed to reach to saturation under conditions which the assay was tested, actin affinity of unacetylated TM was difficult to measure so that it was estimated to be much lower than $0.1x10^6M^{-1}$ [3,15]. This results indicated that the actin affinities of muscle TM and Ala-TM were nearly identical and were

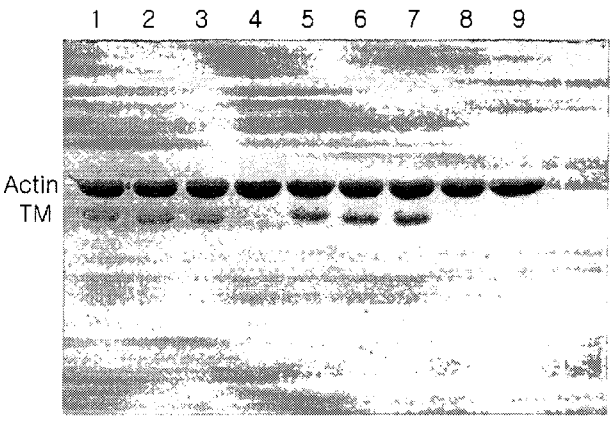

Fig. 2. SDS-polyacrylamide gel analysis of pellets of actin binding assay with tropomyosins.
Conditions: 5 μM actin, 0 - 2.0 μM tropomyosins, in 10 mM imidazole, pH 7.0, 150 mM NaCl, 2.0 mM $MgCl_2$, 0.5 mM DTT.
Lane 1, actin, 1 μM muscle TM; *lane 2*, actin, 1 μM AS-TM; *lane 3*, actin, 1 μM Ala-TM; *lane 4*, actin, 1 μM unacetylated TM; *lane 5*, actin, 2 μM muscle TM; *lane 6*, actin, 2 μM AS-TM; *lane 7*, actin, 2 μM Ala-TM; *lane 8*, actin, 2 μM unacetylated TM; *lane 9*, actin alone.

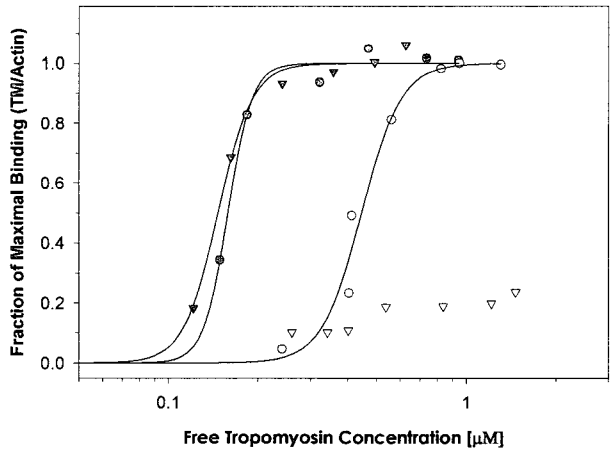

Fig. 3. Binding of tropomyosins to actin.
Conditions: 5 μM actin, varied concentration of tropomyosins, in 10 mM imidazole, pH 7.0, 150 mM NaCl, 2.0 mM $MgCl_2$, 0.5 mM DTT.
Symbols: ● acetylated muscle TM, ○ Ala-TM, ▼ AS-TM, ▽ unacetylated TM.

drastically increased, at least 60 folds, in actin affinity of unacetylated TM, confirming results shown in Fig. 2. However, actin affinity of Ala-TM was still approximately three-fold lower than those of muscle and Ala-Ser-TM. Thus a single residue extension at the amino terminus of tropomyosin was insufficient for complete restoration of tropomyosin function and dipeptide, Ala-Ser extension was

required for high actin affinity.

It has been reported in several occasions that fusion tropomyosins at the amino terminus increased the actin affinity of recombinant tropomyosin [3,5,10,18,20,21,24,31]. Since the longer the fusion peptide tend to show the higher the actin affinity, in some cases even higher than muscle tropomyosin, it was tempting to argue that increase in actin affinity may be somewhat proportional to the length of a fusion peptide at the amino terminus of tropomyosin. Recently, however, Bharadwaj *et al.* [2] reported that HA-TM, hemagglutinin epitope peptide (19 residues) fusion tropomyosin was bound to actin poorly, suggesting that not all fusion tropomyosin was bound well to actin like muscle tropomyosin.

Even though Ala-TM bound to actin weaker than muscle and AS-TM, it was worth to note that simple addition of a single residue to the amino terminus remarkably increase the actin affinity as compared to unacetylated TM. Unless other amino acid residues are tested individually, it is premature to discuss the importance of alanine residue on tropomyosin functions. Difficulties of substituting Ala of Ala-TM to other amino acid residues lie on removal of the initiating Met residue in bacterial expression system. For instance, substitution to Asp residue results in production of dipeptide (Met-Asp) fusion tropomyosin rather than a single amino acid residue extension to the amino terminus. It seems likely that higher actin affinity of Ala-TM may be attributed to the presence of an amino acid residue on the amino terminus rather than the alanine residue *per se* although further subsequent experiments are required for determining role of Ala residue precisely.

Unlike muscle tropomyosin whose amino terminus was blocked by N-acetylation, the amino terminus of unacetylated tropomyosin may be protonated under the physiological and experimental conditions so that a positive charge was present at the amino terminus. It had been proposed that the presence of a positive charge cause deleterious effect on actin affinity, presumably interfering with interaction between the amino and the carboxyl termini in the overlap region of tropomyosin molecules [4-5,21]. Alternatively the unacetylated amino terminus destabilizes the α-helix at the amino terminus of tropomyosin by interfering with the helix dipole but the effect of a positive charge at the amino terminus on stability of the helix dipole was contentious [6-8].

ATPase Regulation Function of TM

Tropomyosin is a regulatory protein of muscle contraction and regulatory function is often determined by an effect on the ATPase. The actin-activated myosin S1 MgATPase, considered an *in vitro* analogue of muscle contraction, was measured as another assay for tropomyosin functions.

A well-known property of tropomyosin is its dual regulatory activity, that is, the ability to inhibit or activate the actomyosin S1 ATPase depending on the ratio of myosin S1 to actin and experimental conditions [17,19]. This cooperative effect is usually interpreted in terms of the myosin head "turning on" the thin filament by causing a change in the position or conformation of tropomyosin on the actin filament [33]. Cho *et al.* [5] showed that skeletal muscle tropomyosin inhibits the ATPase at low ratios of myosin S1 to actin and activated the ATPase activity at higher myosin S1 concentration while NS1 fusion (80 residues) TM inhibited the ATPase even more effectively than muscle tropomyosin at low myosin S1 concentration and the ATPase remained to be inhibitory at all myosin S1 concentration tested.

For analysis of dual regulatory activity, the actomyosin S1 ATPase was measured at low and high concentration of myosin S1 as a function of tropomyosin concentration. As shown in Fig. 4, muscle TM, Ala-TM, and AS-TM inhibited

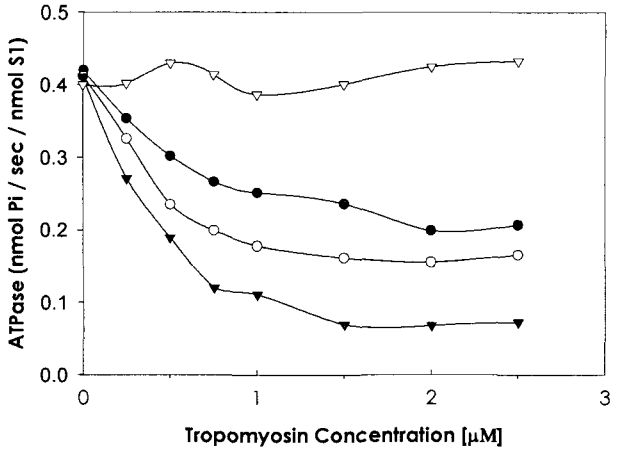

Fig. 4. Comparison of the effects of tropomyosins on the actomyosin S1 ATPase activity measured as a function of tropomyosin concentration at low S1 concentration Conditions: 0.5 μM myosin S1, 5 μM actin, 0-2.5 μM tropomyosins, in 5 mM imidazole, pH 7.0, 40 mM NaCl, 0.5 mM $MgCl_2$, 2 mM MgATP. Symbols: ● acetylated muscle TM, ○ Ala-TM, ▼ AS-TM, ▽ unacetylated TM.

actomyosin S1 ATPase as expected at low myosin S1 concentration. Nevertheless level of the inhibition was different from each other. AS-TM inhibited the ATPase activity approximately 50 percent greater than muscle TM, contrary to previous report that muscle TM and AS-TM were very similar if not identical [21]. Ala-TM inhibited the ATPase more than muscle TM and less than AS-TM but level of the inhibition was closer to muscle TM. Unacetylated tropomyosin had no effect on the actomyosin S1 ATPase, as has been shown for carboxypeptidase-treated tropomyosin [11]. No effect on the ATPase by these tropomyosins would be expected since they bind poorly to actin.

At higher myosin S1 concentration, all tropomyosins increased the ATPase activity as TM concentrations increased as shown in Fig. 5. Level of the ATPase activation was muscle TM > Ala-TM > AS-TM > unacetylated TM in decreasing order. This result showed that muscle and AS differ in regulation of myosin S1 ATPase activity although they have nearly identical actin affinities. In contrast to the result at low S1 concentration, unacetylated TM increased to some extent the ATPase activity at high S1 concentration. This may be attributed to the higher concentration of myosin S1 since it has been reported that myosin S1 increased actin affinity of unacetylated tropomyosin [22].

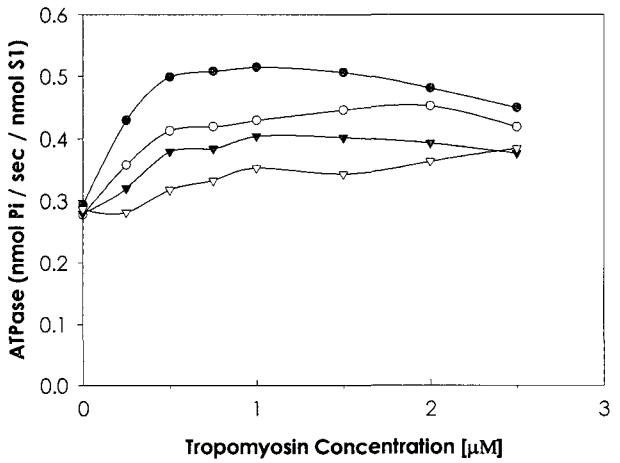

Fig. 5. Comparison of the effects of tropomyosins on the actomyosin S1 ATPase activity measured as a function of tropomyosin concentration at high S1 concentration Conditions: 5 μM myosin S1, 5 μM actin, 0 - 2.5 μM tropomyosins, in 5 mM imidazole, pH 7.0, 40 mM NaCl, 0.5 mM $MgCl_2$, 2 mM MgATP. Symbols: ⊛ acetylated muscle TM, ○ A-TM, ▼ AS-TM, ▽ unacetylated TM.

Since AS-TM apparently inhibited the ATPase greater than Ala-TM and Ala-TM inhibited more than muscle (acetylated) TM, it seemed that the larger in size of modifying group (Ala-Ser, Ala, acetyl group) on the amino terminus of tropomyosin make the effect on the ATPase more inhibitory and prevent the more myosin S1 from switching the thin filament from inhibited state to the potentiated state. Reason for higher inhibition of the ATPase by Ala-TM (or AS-TM) was unclear at this moment and it remained to be studied thoroughly. Nonetheless all recombinant TMs were different from each other in degree of the inhibition of the ATPase activity and only difference in structure was the structure of the amino terminus, these results indicated that the local structure at the amino terminus was crucial for tropomyosin functions.

Acetylation of N-terminus of Proteins

Judged from results of effect of local structure at the amino terminus on the ATPase activity, it is worth to note the importance of acetylation at amino terminus of tropomyosin. Although N-terminal acetylation is a common cotranslational modification of cytoplasmic proteins in eukaryotes [25], the functional significance of this modification has been determined in only a small number of cases. In budding yeast, the three N-terminal acetyltransferases are multiprotein complexes known as NatA, NatB, and NatC [26]. Three related proteins, Ard1p, Nat3p, and Mak3p, appear to be the catalytic subunits of the enzymes, respectively, although other proteins also are required for function. Analysis of the yeast N-terminal acetyltransferases, using knockout strains and an extensive set of substrate specificity has revealed that acetylation is primarily dependent on the amino acid identity of the second (sometimes in combination with the third) residue of a target polypeptide [25-27]. NatB, Nα-terminal acetyltransferase of *Saccharomyces cerevisiae* acts cotranslationally on proteins with Met-Glu or Met-Asp-termini and subclasses of proteins with Met-Asn- and Met-Met-termini. According to these observations, tropomyosin which has an aspartic acid in the second position following methionine residue may be acetylated by Nat3p within the NatB complex. NatB is composed of the interacting Nat3p and Mdm20 subunits, both of which are required for acetyltransferase activity. Recently Singer and Shaw [30] reported that Mdm20p-dependent, N-terminal acetylation of yeast tropomyosin Tpm1p by the Nat B complex is required for Tpm1p association with, and stabilization of, actin fila-

ments and cables.

The results in this study showed that the local structure of the amino terminus was crucial for functions and a simple addition of a residue or dipeptide was insufficient for complete restoration of functions of tropomyosin. Despite a great deal of advantage employing *E. coli* expression system for mass production of eukaryotic proteins, one of the disadvantage is that bacteria are unable to carry out posttranslational modification such as glycosylation because they lack membrane bound organelles. Judged from the results in this paper, glycosylation is not the only problem on the posttranslational modification in bacterial expression system. N-acetylation of proteins, at least in case of tropomyosin, should be considered as an important factor for selecting the choice of overexpression systems.

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초록 : α-Tropomyosin의 아미노 말단 구조가 기능에 미치는 영향

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α-Tropomyosin (TM)의 아미노(N) 말단 구조의 중요성을 확인하기 위하여 N 말단에 알라닌 아미노산 잔기 하나를 첨가한 재조합 Ala-TM을 제조하였다. Ala-TM을 대장균에서 대량발현 시켜 정제한 후, N 말단이 아세틸화된 근육TM 및 N 말단에 알라닌-세린 잔기를 첨가한 AS-TM과 N 말단이 비아세틸화된 TM 등의 재조합 TM과 기능을 비교하였다. Ala-TM은 비아세틸화된 TM보다 액틴친화력이 현저히 증가했으나, 근육 및 AS-TM 보다는 약 3배 정도 약하게 액틴에 결합하였다. 근육 TM, AS-TM, 그리고 Ala-TM 모두가 myosin S1의 농도가 낮을 때 ATPase 활성을 억제하였고 농도가 높을 때 촉진하였으나, 억제와 촉진의 정도는 서로 차이가 있었으며 비아세틸화된 TM은 억제하지 않았다. 이들 결과는 N 말단 구조가 TM의 기능을 결정하는 중요한 요소임을 나타내며 TM의 온전한 기능을 위해서는 아세틸화된 N 말단이 필요하다는 것을 의미한다.