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N-Terminal Modification and Its Effect on the Biochemical Characteristics of Akazara Scallop Tropomyosins Expressed in *Escherichia coli*

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Akazara scallop striated muscle tropomyosin mutants without a fused amino acid (nf-Tm), and with Ala- (A-Tm) or Asp-Ala- (DA-Tm) fused at the N-terminus were expressed in *Escherichia coli* cells. Among them, nf-Tm alone has an initial methionine. The native Akazara scallop tropomyosin and DA-Tm showed similar α-helix contents and intrinsic viscosity, but nf-Tm and A-Tm exhibited lower values than those of the native tropomyosin. According to the relative viscosity, all the expressed tropomyosins appear to have lost head-to-tail polymerization ability. Though nf-Tm has extremely low actin-binding ability, the ability was almost completely recovered with a two amino acid fusion but incompletely with a one amino acid fusion. On the other hand, an amino acid fusion, irrespective of the number, seemed to inhibit the Mg-ATPase activity of actomyosin. However, the bacterially expressed tropomyosins together with Akazara scallop troponin do not confer the full Ca^{2+}-regulation ability of Mg-ATPase activity of actomyosin. These results support that N-terminal blocking probably by an acetyl group of Akazara scallop tropomyosin plays an important role not only in head-to-tail polymerization and actin-binding, as known for vertebrate tropomyosin, but also in maintaining the secondary or higher structure and Ca^{2+}-regulation together with troponin.

Key words: bacterial expression, muscle contraction, scallop, tropomyosin mutants, troponin.

Abbreviations: IPTG, isopropyl-β-D(−)-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; CDTA, *trans*-12-cyclohexanediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; nf-Tm, non-fused Akazara scallop tropomyosin expressed in *Escherichia coli*; A-Tm; Ala-fused Akazara scallop tropomyosin at the N-terminus expressed in *Eschirichia coli*; DA-Tm; Asp-Ala-fused Akazara scallop tropomyosin at the N-terminus expressed in *Escherichia coli*.

Tropomyosins are highly conserved rod-shaped proteins consisting of a double-stranded α-helical coiled-coil structure with molecular weights of approx. 70,000. They form homodimers or heterodimers of two slightly different subunits, and self-polymerize through head-to-tail overlapping under physiological conditions. The vertebrate tropomyosin subunit consists of 284 amino acid residues of approx. 41 nm in length (*1*), and binds to both F-actin and troponin.

The actin-linked tropomyosin-troponin complex regulates the contraction of vertebrate skeletal muscle (*2*). Troponin is a Ca^{2+}-binding protein involved in contraction consisting of three different components, *i.e.* of troponin C, troponin I and troponin T. In skeletal muscle, the contractile interaction of myosin and actin-tropomyosin is depressed by troponin I in the relaxed state, but the contraction is triggered by Ca^{2+}-binding to troponin C. On the other hand, in molluscan muscles such as Akazara scallop and Ezo-giant scallop striated and smooth adductor muscles, contraction is regulated by two Ca^{2+}-regulation systems, *i.e.* troponin-tropomyosin and myosin regulatory light chain (*3*, *4*). It has been demonstrated that scallop striated muscle troponin possesses some different properties from the vertebrate counterpart, *e.g.*, troponin C binds only one Ca^{2+} at site IV out of sites I–IV (*5*–*7*) in contrast to four Ca^{2+} in the case of rabbit troponin C, and troponin I has a more than 130 residues longer N-terminal portion and a considerably larger molecular weight (34,600) than rabbit troponin I (23,000) (*8*). Further, the amino acid sequence of scallop troponin C has been determined, which revealed it shows low homology to vertebrate troponin C.

Recently, Shiraishi *et al.* demonstrated that ATPase was activated at higher Ca^{2+}-concentrations by both myosin regulatory light chain and troponin C, preferably at a lower temperature such as 15°C, as judged after reversible dissociation and association of the light chain and troponin C by means of CDTA-treatment of scallop striated myofibrils. In addition, the activation by troponin C was much greater than that by the regulatory light chain (*9*).

Under these circumstances, we attempted to prepare and characterize various bivalve muscle tropomyosins (*10*). The subunit of these tropomyosins has a molecular weight of approx. 33,000–34,000, and its N-terminus is blocked similarly to other vertebrate and invertebrate

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counterparts. Interestingly, bivalve tropomyosins show quite high viscosity at low salt concentration and strong inhibition of actomyosin Mg-ATPase activity (*10*). Therefore, we determined the amino acid sequences of Akazara scallop (*11*) and Ezo-giant scallop tropomyosins (Nishita *et al*., 1999; GenBank accession number AB004636).

Hitchcock-DeGregori and Heald (*12*) reported that bacterially expressed vertebrate tropomyosins are not functional because of a lack of N-terminal acetylation. In spite of having the same sequence, such a recombinant tropomyosin shows poor actin-binding and head-to-tail polymerization compared to the native tropomyosin. From these results, they proposed that N-terminal acetylation is essential for the tropomyosin function. In contrast, chicken muscle tropomyosin expressed in insect cells exhibits the N-terminal acetylation and indistinguishable functions from those of the native tropomyosin (*13*). On the other hand, Monteiro *et al.* (*14*) reported that *E. coli*-expressed tropomyosin required at least an N-terminal two amino acid fusion for its function.

In the present paper, we describe the construction of Akazara scallop tropomyosin mutants with or without an N-terminal amino acid(s) fusion, and their biochemical characteristics as to head-to-tail polymerization, actin-binding, Ca^{2+}-regulation. Moreover, the effect of N-terminal modification of *E. coli*-expressed Akazara scallop tropomyosin is discussed.

MATERIALS AND METHODS

Construction of Expression Systems—E. coli BL21(DE3) cells (Novagen) and the pET-16b plasmid (Novagen) were used as the host strain and vector DNA for expression, respectively. Akazara scallop tropomyosin cDNA (*11*) was subcloned into pBluescript II KS (+) (Stratagene) and then used as a template DNA for PCR-based mutagenesis. Further, three forward primers, nf-Tm(+) (5′-TCA AAC AAC CAT GGA TGC TAT CAA G-3′), ATm(+) (5′-GAC CAT GGC TAT GGA TGC TAT CAA G-3′), and DATm(+) (5′-GTC CCC ATG GAC GCA ATG GAT GCT ATC AAG-3′), and one reverse primer, TmBam(–) (5′-ATT GCT GGT TAC TAA GGA TCC TCC-3′), were constructed for PCR. After the amplified DNAs had been digested with a mixture of *Nco* I and *Bam*H I, the DNA fragments produced were subcloned into the pET-16b plasmid, and then introduced into *E. coli* BL21(DE3) cells. The nucleotide sequences of both strands of the inserted cDNA were determined using a *Taq* dye primer cycle sequencing kit (Applied Biosystems) and a model 373A DNA sequencer (Perkin Elmer-Applied Biosystems).

Expression and Purification of Recombinant Tropomyosin—Overnight cultures of transformed *E. coli* cells were inoculated into LB medium and then cultivated at 37°C. When the turbidity of the bacterial suspension reached an absorbance value of 0.5 at 660 nm, 0.4 mM IPTG was added and then the incubation was continued for a further 2.5 h. Cells were harvested by centrifugation at 2,500 × *g* for 15 min, and then suspended into 10 volumes of 20 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, and 0.1 mM PMSF. The suspension was frozen and thawed three times at –20°C and 4°C, respectively, and then centrifuged at 100,000 × *g* for 1 h. Then, the supernatant was poured into 2.5 volumes of ethanol and left to stand at 4°C for 4 h. The precipitate formed was collected by centrifugation at 10,000 × *g* for 15 min and then dried at room temperature. The dry powder was suspended in 6 M urea, 0.5 M KCl, 100 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, and 0.1 mM PMSF, and then incubated at 4°C for 8 h, followed by centrifugation at 10,000 × *g* for 15 min. The supernatant obtained was adjusted to pH 4.4 to precipitate recombinant tropomyosin. The precipitate was dissolved in 6 M urea, 10 mM Tris-HCl (pH 7.5), and 5 mM 2-mercaptoethanol, and then applied to a DEAE-Toyopearl 650M column (1.4 × 18 cm). Elution was performed with a linear gradient of 0 to 300 mM KCl. Fractions containing the purified recombinant tropomyosin were pooled, and then dialyzed against 1 mM $NaHCO_3$ and 5 mM 2-mercaptoethanol.

Determination of the N-Terminal Amino Acid Sequence—Recombinant tropomyosin was subjected to Edman degradation to determine the N-terminal amino acid sequence using a 473A protein sequencer (Perkin Elmer-Applied Biosystems).

Proteins—Akazara scallop tropomyosin and troponin were prepared from striated adductor muscle as described previously (*4*, *5*). Myosin and actin were prepared from rabbit skeletal muscle by the methods of Perry *et al.* (*15*), and Spudich and Watt (*16*), respectively. Protein concentrations were determined by the biuret method using bovine serum albumin Fraction V as a standard protein.

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Porzio and Pearson (*17*) using 10% polyacrylamide gels containing 0.1% SDS. The gels were stained with Coomassie Brilliant Blue R-250, and destained with 20% methanol and 7% acetic acid.

Viscosity—Viscosity was measured at 10°C or 15°C with an Ostwald type viscometer in a solution comprising 10 mM Tris-HCl (pH 7.0), 0–400 mM KCl, and various concentrations of tropomyosin.

Circular Dichroism Spectrum—The circular dichroism spectrum of tropomyosin was measured in a solution comprising 20 mM sodium acetate (pH 7.0), 150 mM KCl, and 1.0 mg/ml tropomyosin at various temperatures using a J-700 spectropolarimeter (Jasco). The α-helix content was calculated using the following equation (*18*):

$$\alpha\text{-Helix content } (\%) = ((-[\theta]_{222} + 3{,}000)/39{,}000) \times 100.$$

Binding Assay of Tropomyosin and Actin—Tropomyosin bound to actin was quantitated by the co-sedimentation procedure at 15°C in a solution comprising 20 mM Tris maleate (pH 6.8), 50 mM KCl, 5 mM 2-mercaptoethanol, 20 μM rabbit F-actin, and various concentrations of tropomyosin. The mixture was incubated at 15°C for 30 min, and then centrifuged at 100,000 × *g* for 1 h. The precipitate thus formed was rinsed three times with the same buffer. After the resulting supernatant and precipitate had been subjected to SDS-polyacrylamide gel electrophoresis, the amounts of bound and unbound tropomyosin to F-actin were estimated from the absorbance at 560 nm with a Chromatoscanner CS-9000 (Shimadzu). The Hill coefficient and apparent binding constant were calculated using the following equation:

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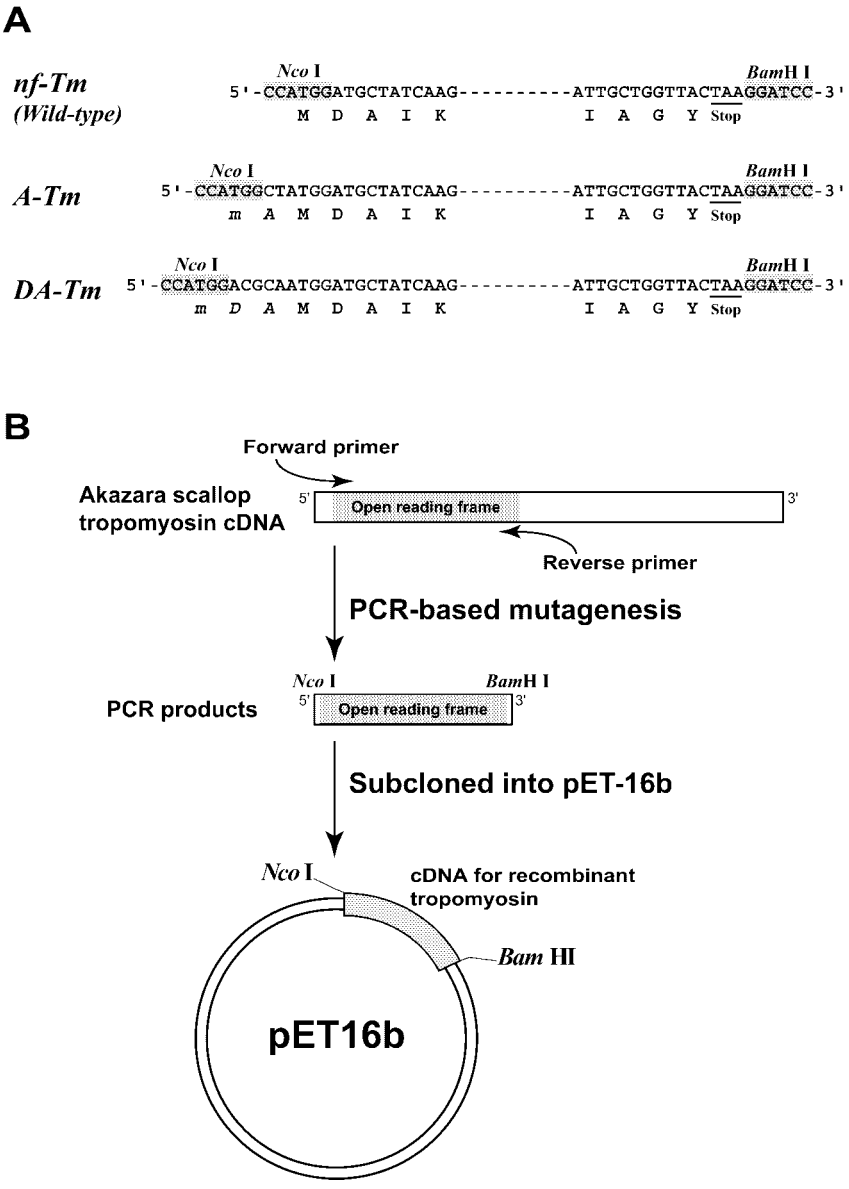

Fig. 1. **Strategy for bacterial expression of Akazara scallop tropomyosins.** A: Nucleotide sequences of the 5′-terminal and 3′-terminal portions of Akazara scallop recombined tropomyosin cDNAs. The first methionine in both A-Tm and DA-Tm was not detected on amino acid sequence analysis probably due to processing in *Escherichia coli*. B: Construction strategy for recombined plasmids comprising pET-16b and the Akazara scallop recombined tropomyosin cDNAs.

$$v = n[\mathrm{Tm}]^H/(K_{app}{}^H + [\mathrm{Tm}]^H)$$

where v is the density ratio of Tm/actin at the concentration of unbound tropomyosin [Tm], n the maximal density ratio of Tm/actin, H the Hill coefficient, and K_{app} the apparent binding constant.

Mg-ATPase Activity—Reconstituted rabbit actomyosin Mg-ATPase activity was measured at 15°C in a solution comprising 20 mM Tris maleate (pH 6.8), 50 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 0.1 mg/ml rabbit myosin, 0.05 mg/ml rabbit F-actin, various concentrations of tropomyosin and troponin, and 0.2 mM EGTA (in the absence of Ca^{2+}) or 0.2 mM EGTA plus 0.3 mM $CaCl_2$ (in the presence of Ca^{2+}). Inorganic phosphate liberated was determined by the method of Youngburg and Youngburg (*19*).

RESULTS

Preparation of Expressed Tropomyosin Mutants—PCR-based mutagenesis was performed using a template of pBluescript II KS(+) recombined with Akazara scallop tropomyosin cDNA (*11*). After DNA fragments for expression had been amplified, they were recombined with the pET-16b plasmids and then introduced into *E. coli* BL21(DE3) cells, as shown in Fig. 1. Then, the Akazara scallop tropomyosin mutants were expressed in the transformed *E. coli* by IPTG induction. The expressed tropomyosins were extracted from the bacteria as described under " MATERIALS AND METHODS" (Fig. 2A). The crude tropomyosin mutants were purified independently by DEAE-Toyopearl 650M column chromatography with a linear gradient of 0 to 300 mM KCl (Fig. 2B). Fractions containing purified recombinant tropomyosin were pooled, and then dialyzed against 1 mM $NaHCO_3$ and 5 mM 2-mercaptoethanol (Fig. 2C). Accordingly, three recombinant tropomyosins, *i.e.* nf-Tm, A-Tm, and DA-Tm, were obtained. The yield of each expressed tropomyosin was about 30 mg from 1,000 ml culture in Luria-Bertani's broth. Amino acid sequence analyses revealed that nf-Tm alone possesses an initiation methionine at the N-

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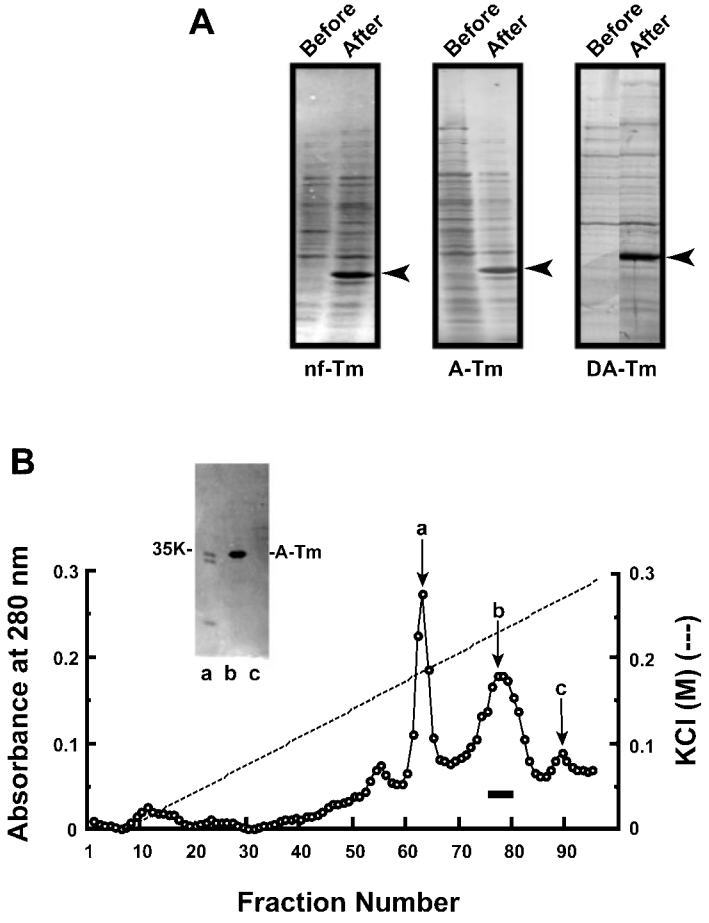

Fig. 2. **Expression and purification of bacterially expressed tropomyosins.** A: SDS-PAGE patterns of whole lysates of *Escherichia coli*. "Before" and "After" show whole lysate before induction by IPTG and 2.5 h after addition of 0.4 mM IPTG, respectively. Arrowheads indicate expressed tropomyosins. B: DEAE-Toyopearl column chromatography of A-Tm. Crude expressed tropomyosin was dialyzed against 6 M urea, 10 mM Tris-HCl (pH 7.5), and 5 mM 2-mercaptoethanol, and then applied to a DEAE-Toyopearl 650M column (1.4 × 18 cm). Elution was performed with a linear KCl gradient, from 0 to 300 mM. Each fraction was 5 ml. The SDS-PAGE patterns of the fractions indicated by arrows (a–c) are shown in the inset. The fractions indicated by a solid bar were pooled. *C,* SDS-PAGE patterns of purified bacterially expressed tropomyosins.

terminus among the three mutant species. Similar processing has been reported for expressed α-tropomyosin in *E. coli* (*14*).

Polymerization of Expressed Tropomyosins—As reported previously, native Akazara scallop tropomyosin derived from striated muscle shows seven times higher relative viscosity than rabbit tropomyosin at various salt concentrations, probably due to head-to-tail polymerization (*10*). Therefore, the relative viscosity of the expressed tropomyosins was measured (Fig. 3). All the expressed tropomyosins showed considerably lower viscosity than Akazara scallop tropomyosin derived from striated muscle in the presence of 200 mM KCl. In the absence of KCl, nf-Tm, A-Tm, and DA-Tm showed relative viscosity of 1.1, 1.7, and 1.7, respectively, while the value for Akazara scallop tropomyosin was 14.5. These results suggest that N-terminal one or two amino acid(s) extension caused a slight increase in polymerization activity but not sufficient recovery for Akazara scallop tropomyosin.

Next, the reduced viscosity of the expressed tropomyosins was measured. As shown in Fig. 4, Akazara scallop tropomyosin and DA-Tm exhibited a considerably steep increase and gradual increase, respectively, with protein concentration. However, nf-Tm and A-Tm exhibited low and constant reduced viscosity. Akazara scallop tropomyosin and DA-Tm showed almost identical intrinsic viscosity values of 0.40 (Table 1) in contrast to those of nf-Tm and A-Tm, *i.e.* 0.20 and 0.25, respectively. Therefore, the molecular shape of nf-Tm and A-Tm is supposed to be more globular than that of Akazara scallop tropomyosin and DA-Tm. Thus, it appears that N-terminal blocking and a two amino acid fusion play a role in stabilizing the secondary or higher structure of Akazara scallop tropomyosin, but less than a one amino acid fusion does not.

α-Helix Content of Expressed Tropomyosins—The α-helix contents of the expressed tropomyosins were calcu-

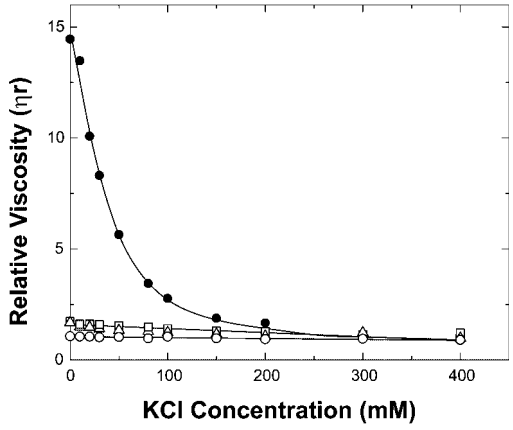

Fig. 3. **The effect of the KCl concentration on the viscosity of expressed tropomyosins.** Viscosity was assayed in a medium comprising 10 mM Tris-HCl (pH 7.0), various concentrations of KCl, and 1.0 mg/ml tropomyosin at 15°C. Closed circles, Akazara scallop tropomyosin; open circles, nf-Tm; triangles, A-Tm; squares, DA-Tm.

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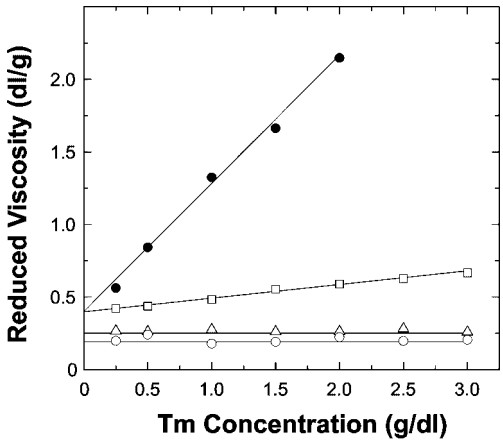

Fig. 4. **Reduced viscocity of expressed tropomyosins.** Viscosity was assayed in a medium comprising 10 mM Tris-HCl (pH 7.0), 100 mM KCl, and various concentratios of tropomyosins at 10°C. Closed circles, Akazara scallop tropomyosin, open circles, nf-Tm; triangles, A-Tm; squares, DA-Tm.

Table 1. **Intrinsic viscosities of tropomyosins.**

Protein	Intrinsic viscosity (dl/g)
Akazara-Tm*	0.40
nf-Tm	0.20
A-Tm	0.25
DA-Tm	0.40

*Native Akazara scallop striated muscle tropomyosin.

lated from the CD-spectrum measured in the presence of 150 mM KCl (Fig. 5). All the expressed tropomyosins showed the maximum contents at 4°C, *e.g.*, 93% for Akazara scallop tropomyosin and DA-Tm. Interestingly, the α-helix contents of the expressed tropomyosins decreased at 4°C as the fused amino acid number decreased, *e.g.*, 80% and 68% for A-Tm and nf-Tm, respectively. These results show a similar tendency to that of the intrinsic viscosity shown in Table 1, suggesting that the N-terminal modification stabilized the secondary structure. In fact, a two amino acid fusion was more effective than a one amino acid fusion for recovery of the higher α-helical structure of native Akazara scallop tropomyosin. In the *inset* in Fig. 5, the decreasing α-helix content is plotted against the temperature change. As can be seen, the maximum decrease was observed at 30°C for Akazara scallop tropomyosin in contrast to 27.5°C for DA-Tm, A-Tm, and even nf-Tm. Therefore, it appears that the N-terminal fusion of one or two amino acid(s) contributes not only to structural stabilization but also to heat resistance against structural change.

Actin-Binding of Expressed Tropomyosins—The expressed tropomyosins were mixed independently with rabbit F-actin in various molar ratios in a 50 mM KCl solution, and then their mutants in the precipitates and supernatant obtained on centrifugation were estimated. As shown in Fig. 6, the various tropomyosins bound to F-actin. The K_{app} value for Akazara scallop tropomyosin was calculated to be 1.5×10^7 M^{-1}, which is approximately 10 times higher than that of vertebrate muscle tropomyosins (*20*, *21*) but comparable to that (4.4×10^7 M^{-1}) of lobster fast muscle type tropomyosin (*22*). DA-Tm and A-Tm bound to F-actin maximally in an equimolar

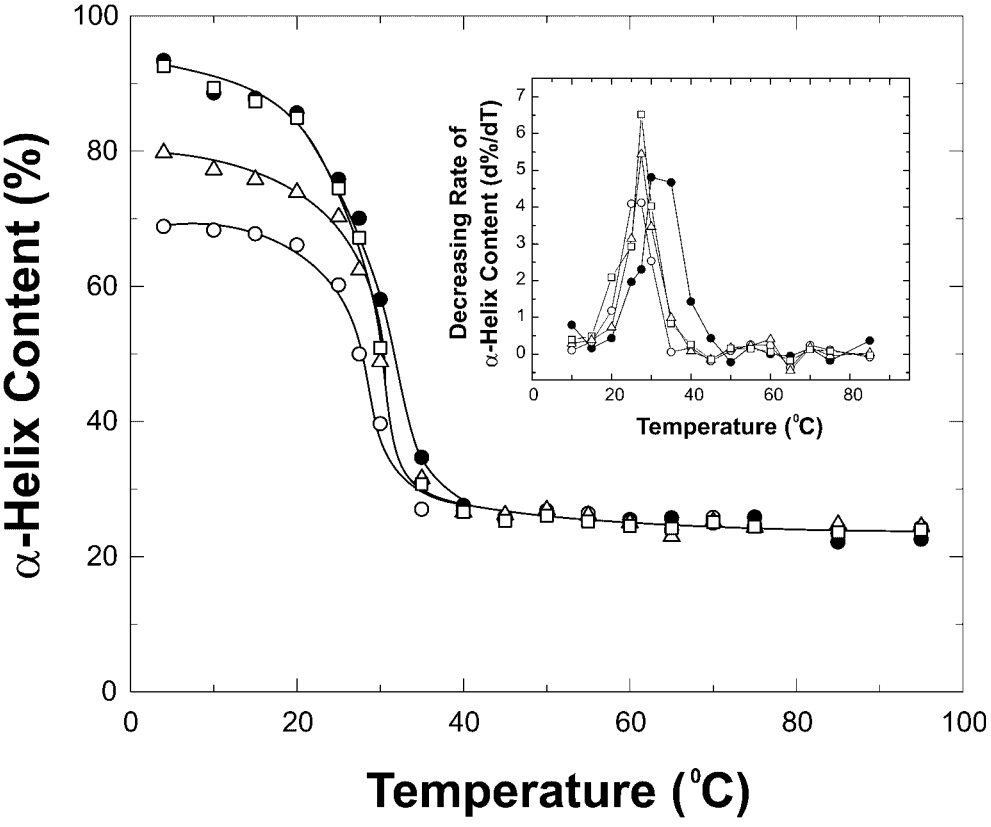

Fig. 5. **Temperature dependence of the α-helix content in expressed tropomyosins.** CD spectra of tropomyosins were measured in a medium comprising 20 mM sodium acetate (pH 7.0), 150 mM KCl, and 1.0 mg/ml tropomyosin. The α-helix content was calculated as described under "MATERIALS AND METHODS." Inset shows the decreasing rate of α-helix content. Closed circles, Akazara scallop tropomyosin; open circles, nf-Tm; triangles, A-Tm; squares, DA-Tm.

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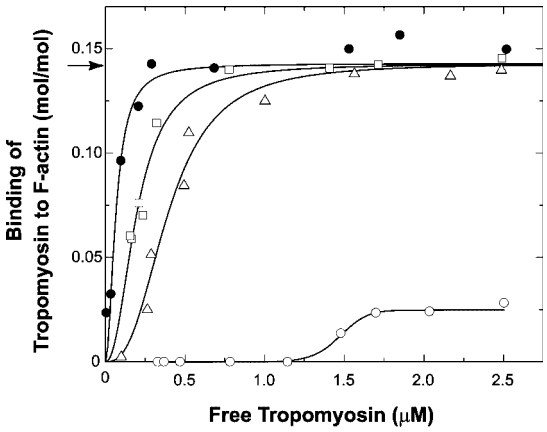

Fig. 6. **Actin binding of expressed tropomyosins.** The arrow indicates the position of the molar ratio of tropomyosin and actin of 1:7. The binding assay was performed in a medium comprising 20 mM Tris maleate (pH 6.8), 50 mM KCl, 5 mM 2-mercaptoethanol, 20 μM rabbit skeletal muscle F-actin, and various concentrations of tropomyosin at 15°C. Closed circles, Akazara scallop tropomyosin; open circles, nf-Tm; triangles, A-Tm; squares, DA-Tm.

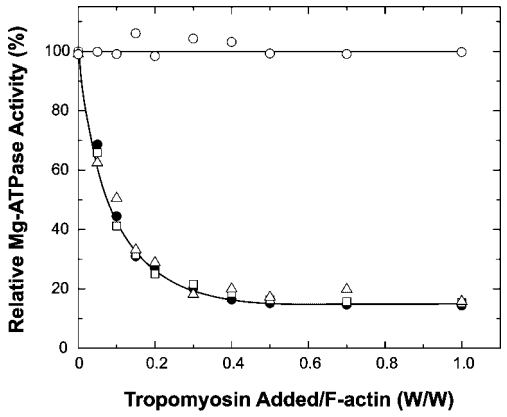

Fig. 7. **Effects of expressed tropomyosins on rabbit actomyosin Mg-ATPase activity.** The ATPase activity was assayed in a medium comprising 20 mM Tris maleate (pH 6.8), 50 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 0.1 mg/ml rabbit skeletal muscle myosin, 0.05 mg/ml rabbit skeletal muscle F-actin, and various concentrations of tropomyosin at 15°C. Closed circles, Akazara scallop tropomyosin; open circles, nf-Tm; triangles, A-Tm; squares, DA-Tm. 100 % activity is equivalent to 142 nmol Pi/min·mg myosin.

amount, however, the K_{app} values of 2.5×10^6 M^{-1} and 5.1×10^6 M^{-1} for DA-Tm and A-Tm, respectively, are lower than that of native tropomyosin. The Hill coefficients for Akazara scallop tropomyosin, A-Tm, and DA-Tm are 1.9, 2.7, and 2.2, respectively, showing that there is no great difference among them. On the other hand, nf-Tm hardly bound to F-actin in the range so far investigated. Such an extremely low actin affinity tropomyosin was observed when vertebrate tropomyosin was expressed in *E. coli*. Although Monteiro *et al.* (*14*) reported that the actin-binding stoichiometry could be recovered for vertebrate tropomyosin expressed in *E. coli* by adding two N-terminal amino acids, our results for scallop tropomyosin expressed in *E. coli* suggest that the addition of only one amino acid is enough for its recovery.

Inhibition of Actomyosin Mg-ATPase Activity by Expressed Tropomyosins—As reported previously (*10*), when scallop tropomyosin was added to rabbit actomyosin under physiological conditions, actomyosin Mg-ATPase activity was inhibited to about 18% of the original level with a ratio of scallop tropomyosin to actin of 1:7 or more (mol/mol). Here, we examined the inhibition of the Mg-ATPase activity of rabbit reconstituted actomyosin by the expressed tropomyosins. As shown in Fig. 7, A-Tm and DA-Tm exhibited similar extents of inhibition to Akazara scallop tropomyosin from muscle, while nf-Tm entirely lost the inhibition ability. These results well agreed with those of the actin-binding experiments (Fig. 6).

Effects of Expressed Tropomyosin and Troponin on Actomyosin Mg-ATPase Activity—To determine whether or not the expressed Akazara scallop tropomyosins have a regulatory function, the Mg-ATPase activity of rabbit actomyosin**,** which was reconstituted from rabbit actin and myosin in the weight ratio of 1:2, together with Akazara scallop troponin and expressed tropomyosin was measured in the presence and absence of Ca^{2+} under physiological conditions. When native scallop tropomyosin was added in various amounts to mixtures of rabbit actomyosin and scallop troponin, the Mg-ATPase activity was repressed to about 10 % as the amount of tropomyosin-troponin complex increased in the absence of Ca^{2+} but not at all in the presence of Ca^{2+}. Thus, the Mg-ATPase activity was regulated effectively (Fig. 8A). Similar results were obtained using nf-Tm–troponin complex, except that the Mg-ATPase activity in the absence of Ca^{2+} was repressed to about 40% (Fig. 8B). In addition, nf-Tm required twice the amount of native tropomyosin for maximum inhibition, probably because of its low affinity to F-actin, as shown in Fig. 6. Therefore, it is assumed that nf-Tm alone has poor head-to-tail polymerization ability (Fig. 3) and F-actin binding ability (Figs. 6 and 7), but partly recovers the F-actin-binding ability and Ca^{2+} regulation ability if troponin coexists (Fig. 8B). On the other hand, A-Tm and DA-Tm showed identical Ca^{2+} regulation features as to actomyosin Mg-ATPase activity (Fig. 8C and D). These two tropomyosin-troponin complex species inhibited the activity to about 10% in the absence of Ca^{2+} similarly to native Akazara scallop tropomyosin-troponin complex. However, they decrease the inhibition only to about 40% in the presence of Ca^{2+}. These facts may suggest that A-Tm and DA-Tm bind to F-actin but hardly move on its surface. Thus, the N-terminal blocking and amino acid fusion of Akazara scallop tropomyosin affect the manner of inhibition and its inhibition of the actomyosin Mg-ATPase.

DISCUSSION

In order to determine the importance of the N-terminal blocking of Akazara scallop striated muscle tropomyosin, we prepared one and two amino acid(s) fused tropomyosin mutants using bacterial expression, and then examined some hydrodynamic and biochemical properties.

On comparison of the Akazara scallop tropomyosin with these mutants, it becomes apparent that the loss of the N-terminal acetylation causes a complete loss of head-to-tail polymerization and actin-binding ability. The Asp-Ala-fusion hardly restored the head-to-tail

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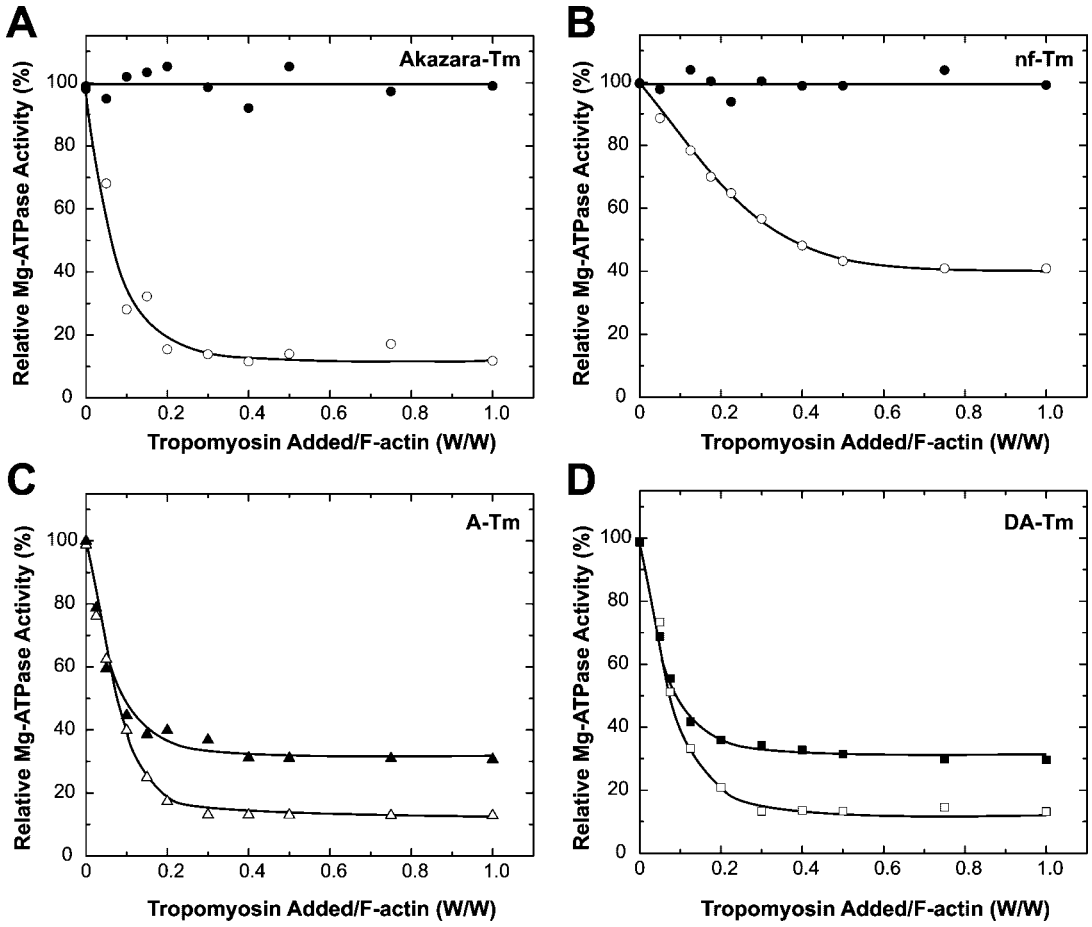

Fig. 8. **Effects of tropomyosin and troponin on Ca^{2+}-regulation of rabbit actomyosin Mg-ATPase activity.** The activity was assayed in a medium comprising 20 mM Tris maleate (pH 6.8), 50 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 0.2 mM EGTA (in the absence of Ca^{2+}, open symbols) or 0.2 mM EGTA and 0.3 mM $CaCl_2$ (in the presence of Ca^{2+}, closed symbols), 0.1 mg/ml rabbit myosin, 0.05 mg/ml rabbit F-actin, 0.025 mg/ml Akazara scallop troponin, and various concentrations of tropomyosin at 15°C. Panels A, B, C, and D show the Mg-ATPase activity as a function of the weight ratios of Akazara scallop tropomyosin, nf-Tm, A-Tm, and DA-Tm, respectively, to F-actin. 100 % activity is equivalent to 128 nmol Pi/min mg myosin.

polymerization but completely restored the actin-binding ability. Hitchcock-DeGregori and Heald (*12*) also reported that bacterially expressed vertebrate α-tropomyosin lost the head-to-tail polymerization ability. However, the molecular mechanism underlying head-to-tail polymerization of scallop tropomyosin appears to be somewhat different from that of a vertebrate counterpart since Ala- or Asp-Ala–fused Akazara scallop tropomyosin increased the relative viscosity to a maximum of 1.7 times, *e.g.*, one ninth the value of native tropomyosin in the absence of KCl. In addition, tropomyosin prepared from bivalve muscles shows high relative viscosity (*10*). The amino acid sequence revealed that the C-terminal nine residues of scallop tropomyosins (GenBank, accession numbers AB021681, AB000907, and AF216520) are completely different from those of vertebrate muscle tropomyosin, though the N-terminal nine residues are completely the same. Therefore, the fusion of only two amino acids might be insufficient to restore the polymerization ability in the case of Akazara scallop tropomyosin. On the other hand, Monteiro *et al*. reported that two amino acid Ala-Ser-fused vertebrate tropomyosin showed completely restored polymerization ability and actin-binding ability (*14*). Moreover, three amino acid (Ala-Ser-Arg- or Ala-Ala-Ser-) fused tropomyosins showed much stronger polymerization ability than native tropomyosin (*13*, *14*). These results support that the N-terminal longer extension causes restoration of the polymerization ability of bacterially expressed tropomyosin similarly to expressed scallop tropomyosin. The reason for the discrepancy between the results of Monteiro *et al*. and ours needs to be investigated further but the possibility of the charge difference between the N-terminal residues of Ala and Asp cannot be excluded.

We have observed that the loss of N-terminal acetylation causes an incomplete loss of the α-helix content and a two amino acid fusion almost completely restores it. Further, judging from the intrinsic viscosity, DA-Tm alone is considered to possess a similar molecular shape to native tropomyosin.

Thus, the N-terminal two amino acid fusion seemed to stabilize the secondary and higher structures. On the other hand, it has been reported that unacetylated full β-tropomyosin and its mutant lacking residues 254–284 (*23*) of human skeletal muscle were expressed in *E. coli* and shown to have an indistinguishable α-helix content

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from the native one (*24*). Greenfield *et al*. observed that N-terminal acetylation stabilized the α-helix structure of a synthetic peptide, TM32, which consists of residues 1–30 of rabbit tropomyosin and C-terminal extension Gly-Cys. However, the acetylation has only a little effect on the overall stability of tropomyosin (*25*).

Native Akazara scallop tropomyosin derived from striated muscle showed strong inhibition of rabbit actomyosin Mg-ATPase activity compared with vertebrate tropomyosin (*10*). Bacterially expressed scallop tropomyosins also showed practically the same inhibition of actomyosin Mg-ATPase as vertebrate tropomyosin. Monteiro *et al*. reported that two (Ala-Ser-) or three (Ala-Ala-Ser-) amino acid fused tropomyosin inhibited actomyosin Mg-ATPase activity, but non-fused tropomyosin completely lacked the inhibitory activity (*14*). In the present study, one amino acid fused tropomyosin A-Tm also resulted in indistinguishable inhibition of actomyosin Mg-ATPase activity.

All the expressed scallop tropomyosins showed inadequate Ca^{2+}-regulation of actomyosin Mg-ATPase activity together with troponin. In contrast, non-fused or more than two amino acid fused vertebrate tropomyosin exhibited Ca^{2+}-regulation together with troponin like muscle tropomyosin (*14*). These results suggest that the inhibition of Mg-ATPase activity by tropomyosin is a primarily important event for Ca^{2+}-regulation through the tropomyosin-troponin system in scallop striated muscle.

In addition, the unique structures and functions of scallop troponin subunits should be taken into consideration. As reported previously, scallop troponin T showed relatively low amino acid sequential homology (26%) with rabbit troponin T having an extended and acidic-rich region consisting of 76–83 residues at the C-terminus (*26*, *27*). Troponin I also has an N-terminal extended domain which has never been found in vertebrate troponin I (*8*). In addition, troponin C can bind only one mole Ca^{2+}/mol at site IV (*6*, *7*).

From these results, we conclude that the roles of N-terminal blocking by the acetyl group of scallop tropomyosin are to enhance not only head-to-tail polymerization and actin-binding, as known for vertebrate tropomyosin, but also maintenance of the secondary or higher structure so as to inhibit actomyosin Mg-ATPase activity and regulate the muscle contraction together with troponin.

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