

Functional α -Tropomyosin Produced in *Escherichia coli*

A DIPEPTIDE EXTENSION CAN SUBSTITUTE THE AMINO-TERMINAL ACETYL GROUP*

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Unlike the muscle protein, α -tropomyosin expressed in *Escherichia coli* does not bind actin, does not exhibit head-to-tail polymerization, and does not inhibit actomyosin ATPase activity in the absence of troponin. The only chemical difference between recombinant and muscle tropomyosins is that the first methionine is not acetylated in the recombinant protein (Hitchcock-DeGregori, S. E., and Heald, R. W. (1987) *J. Biol. Chem.* 262, 9730–9735). We expressed three fusion tropomyosins in *E. coli* with 2, 3, and 17 amino acids fused to its amino terminus. All three fusions restored actin binding, head-to-tail polymerization, and the capacity to inhibit the actomyosin ATPase to these unacetylated tropomyosins. Unlike larger fusions, the small fusions of 2 and 3 amino acids do not interfere with regulatory function. Therefore the presence of a fused dipeptide at the amino terminus of unacetylated tropomyosin is sufficient to replace the function of the *N*-acetyl group present in muscle tropomyosin. A structural interpretation for the function of the acetyl group, based on our results and the coiled coil structure of tropomyosin, is presented.

Tropomyosin is a filamentous protein composed of two predominantly α -helical chains arranged parallel and in register (for a review, see Smillie, 1979). In the thin filaments of muscle, tropomyosin molecules lie along the actin helix and are joined end to end via overlaps at their amino and carboxyl termini. Together with troponin, tropomyosin forms part of a calcium-sensitive switch that regulates muscle contraction (for a review, see Zot and Potter, 1987).

Analysis of the tropomyosin primary sequence revealed a heptad repeat that extends throughout the entire 284-amino acid sequence. In each successive group of seven residues, *a*–*g*, residues *a* and *d* ("core" residues) are hydrophobic. Hydrophobic interactions between parallel strands stabilize the coiled coil structure of tropomyosin. Salt bridges between charged residues in the "inner" positions *e* and *g* appear to add further stability and specificity of chain interaction to the coiled coil structure (for reviews, see Smillie, 1979 and Phillips *et al.*, 1986).

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Muscle α -tropomyosin has been expressed in *Escherichia coli* as nonfusion and fusion proteins (Hitchcock-DeGregori and Heald, 1987). The amino acid sequence of recombinant nonfusion tropomyosin is identical to muscle tropomyosin with the difference that the first methionine is not acetylated (Hitchcock-DeGregori and Heald, 1987). This unacetylated tropomyosin binds poorly to actin and does not polymerize, suggesting that the charge at the amino terminus may be critical for tropomyosin function. A fusion tropomyosin with 80 amino acids fused to the amino terminus binds actin but is nonpolymerizable and does not promote regulation of the actomyosin Mg^{2+} -ATPase by troponin (Hitchcock-DeGregori and Heald, 1987). We have reported previously the expression of chicken skeletal muscle tropomyosin in *E. coli* as an amino-terminal fusion containing 31 amino acids of bacteriophage λ cII protein (Iserhardt and Reinach, 1988). This protein, binds to F-actin in a centrifugation assay.¹

Here we show that the addition of a di- or tripeptide at the amino terminus of recombinant tropomyosin can substitute the function of the *N*-acetyl group present on muscle tropomyosin. These fusion recombinant tropomyosins bind to actin, polymerize, and are capable of regulating the actomyosin ATPase.

MATERIALS AND METHODS

Construction of Tropomyosin Expression Vectors—The T7-based pET expression system (Studier *et al.*, 1990) was used to express tropomyosin (Tmy).² A *NcoI*-*HindIII* fragment containing the complete coding sequence of chicken skeletal muscle α -tropomyosin (Gooding *et al.*, 1987) was subcloned in pET-3d to construct pET-Tmy for expression of nonfusion tropomyosin (nfTmy). To produce a fusion tropomyosin with 18 amino acids at the amino terminus (S10FXTmy), we isolated the *Bam*HI-*Hind*III fragment of M13mp19-clIFXTmy (Iserhardt and Reinach, 1988), which contains the FX sequence fused to the 5' side of the Tmy gene and cloned it in pET-3a in-frame with the first 11 codons for the T7 gene 10 protein (S10). To reduce the amino-terminal fusion peptide to 4 and 3 amino acids, *NdeI* and *NheI* sites were introduced into the FX sequence using oligonucleotide-mediated site-directed mutagenesis (Kunkel, 1987) (Fig. 1A). Initially the *Bam*HI-*Hind*III fragment of pET-S10FXTmy was subcloned into M13mp19 constructing M13mp19-FXTmy. This vector was mutated with the oligonucleotide 2RS (5'-CCGGGGATCCCATATGGCTGCTAGCATGGATGCCATC-3') to produce the construct M13mp19-2RSTmy. The sequence of mutated Tmy cDNA was confirmed by dideoxy DNA sequencing (Sanger *et al.*, 1977). The *NheI*-*Hind*III fragment of M13mp19-2RSTmy was cloned into pET-3a constructing pET-MASTmy to code for Tmy with 3 amino acids (Met-Ala-Ser) fused to its amino terminus. The *NdeI*-*Hind*III frag-

¹ S. V. Iserhardt and F. C. Reinach, unpublished results.

² The abbreviations used are: Tmy, tropomyosin; nfTmy, nonfusion tropomyosin; ASTmy, fusion tropomyosin with 2 extra amino acids (Ala-Ser) on its amino terminus; AASTmy, fusion tropomyosin with 3 extra amino acids (Ala-Ala-Ser) on its amino terminus; S10FXTmy, fusion tropomyosin containing 11 amino acids of bacteriophage T7 gene 10 protein (S10) and 6 amino acids of the factor Xa cleavage site (FX) on its amino terminus; mTmy, muscle Tmy; DTT, dithiothreitol; S1, myosin subfragment 1; Tn, troponin.

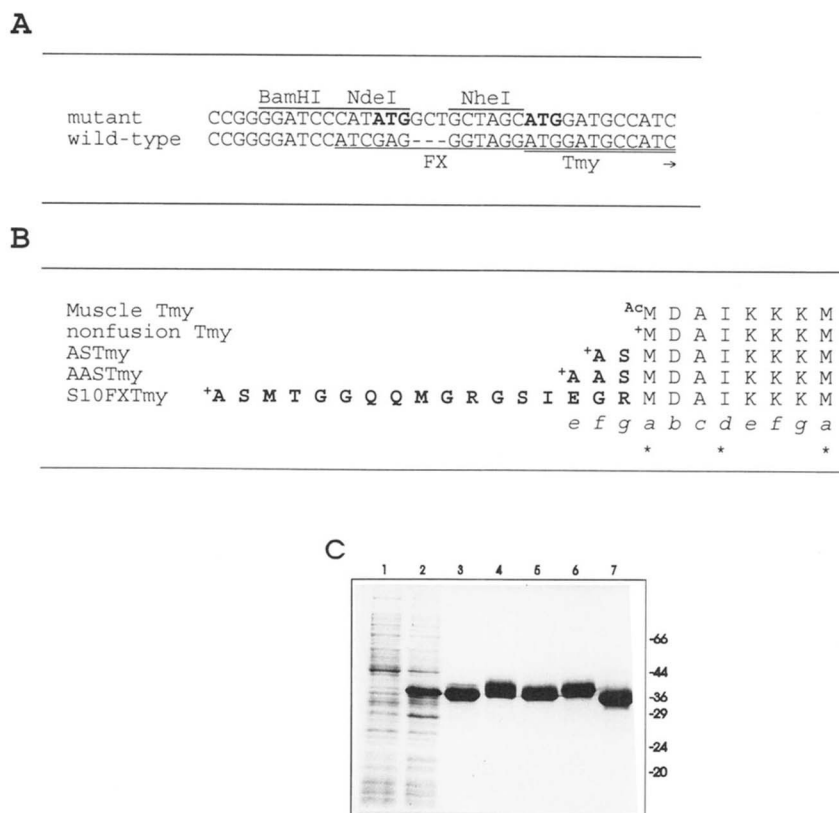


FIG. 1. *Panel A*, diagram of the region subjected to site-directed mutagenesis. The region of FX (*underlined*) and Tmy (*double underlined*) sequences used for site-directed mutagenesis and the sequence of the mutagenic oligonucleotide 2RS are *aligned*. The positions of the restriction sites created to construct the different fusion proteins are shown. The initiation methionine codons are shown in *bold*. *Panel B*, comparison of amino-terminal sequences of acetylated mTmy, nTmy, and the three fusion tropomyosins produced in this study. The *bottom* portion of the figure shows the position in the heptapeptide repeats (positions *a-g*) for each residue of the tropomyosin molecule. Asterisks mark the positions of the hydrophobic residues. Observe that in recombinant Tmy the charged Met-1 occupies an *a* position, which is restricted to hydrophobic residues in the coiled coil. The fusion peptides are in *bold*. *Panel C*, SDS-polyacrylamide gel showing the level of expression and the purified tropomyosin from *E. coli*. Total extracts of bacteria harboring the pET-Tmy expression plasmid before (*lane 1*) and after induction (*lane 2*); purified nonfusion tropomyosin (*lane 3*); purified ASTmy (*lane 4*); purified AASTmy (*lane 5*); purified S10FXTmy (*lane 6*); and purified mTmy (*lane 7*). Molecular masses of protein standards are indicated in kDa. The samples were run on SDS-12.5% polyacrylamide gels and stained with Coomassie Blue. Tropomyosin ($M_r = 33,000$) migrates anomalously with an apparent molecular weight of about 36,000.

ment of the M13mp19-2RSTmy was cloned into pET-3a to produce pET-MAASTmy, which codes for Tmy with 4 amino acids (Met-Ala-Ala-Ser) fused to its amino terminus.

Purification of Recombinant Tropomyosins and Their Amino-terminal Sequence Determination—For expression we used *E. coli* strain BL21(DE3) pLysS since these constructs are not stable in leaky strains such as BL21(DE3). A single colony from a fresh plate was inoculated into 50 ml of 2 × TY (16 g/liter Tryptone, 10 g/liter yeast extract, 5 g/liter NaCl, pH 7.4) with 100 µg/ml of carbenicillin and 200 µg/ml of chloramphenicol and grown for 3 h at 37 °C. A liter of 2 × TY (plus antibiotics) was inoculated with 10 ml of this culture and grown until $A_{600\text{ nm}} = 0.8$. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.4 mM, and incubation was continued for another 3 h. Cells were collected by centrifugation (4,500 × *g*, 10 min, 4 °C), frozen, thawed, and resuspended in 20 ml of 50 mM Tris-HCl (pH 8.0), 25% (w/v) sucrose, 1 mM EDTA and lysed in a French pressure cell press (16,000 p.s.i.). The lysate was cleared (10,000 × *g*, 20 min, 4 °C), and 3 volumes of ethanol were added to the supernatant. The precipitate that formed overnight at room temperature was collected by centrifugation (10,000 × *g*, 20 min, 4 °C), washed with 4 volumes of ethanol, and dried. The ethanol powder was extracted with 50 mM Tris-Cl (pH 7.5), 1 M KCl, 14 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride at 4 °C for 4 h with gentle stirring. After extraction, the preparation was cleared (10,000 × *g*, 20 min, 4 °C), and the pH of the supernatant was decreased slowly to 4.6 with 1 N HCl on ice with gentle stirring for another 20 min. The precipitate obtained by centrifugation (8,000 × *g*, 15 min, 4 °C) was resuspended in 1 mM DTT, and the pH was adjusted to 7.0 with 1 N NaOH. Solid ammonium sulfate was added to 40% saturation, the pH adjusted to 7.0, and the preparation was stirred for 20 min at 4 °C. After centrifugation (16,000 × *g*, 30 min, 4 °C) solid ammonium sulfate was added to the supernatant to 70% saturation. Precipitated proteins were collected (16,000 × *g*, 30 min, 4 °C), resuspended in 20 ml of 50 mM

Tris-Cl (pH 8.0), 0.01% Na₂S₂O₃, 0.5 mM EDTA, 14 mM β-mercaptoethanol, and dialyzed against the same buffer. Urea was added to 8 M final concentration, and the proteins were loaded onto a 100-ml DEAE-cellulose column equilibrated with the same buffer (20 ml/h, room temperature). The column was washed with the same buffer, and Tmy was eluted with a 0–250 mM NaCl gradient (360 ml, 20 ml/h). The Tmy fractions were pooled, dialyzed first against 50 mM Tris-Cl (pH 7.5), 150 mM KCl, and 1 mM DTT to remove urea, then against 1 mM DTT, 1 mM NH₄HCO₃ before freeze drying. Amino-terminal amino acid sequencing of pure proteins was done by Edman degradation on an Applied Biosystems model 473A protein sequenator.

Purification of Muscle Proteins—Troponin was purified from pectoralis muscle of adult chickens according to Ebashi *et al.* (1971). Actin was purified from acetone powder of chicken pectoralis major and minor muscles according to the protocol of Pardee and Spudich (1982). Muscle α-tropomyosin was purified from adult chicken heart according to Smilie (1982). Myosin was purified from chicken pectoralis major and minor muscles according to Reinach *et al.* (1982), and S1 was prepared according to Margossian and Lowey (1982).

The following extinction coefficients were used to calculate protein concentrations: $E_{280}^{1\%} = 7.9$ for S1 and $E_{278}^{1\%} = 2.9$ for all tropomyosins. The F-actin concentration was calculated from the absorbance at 290 and 320 nm using the relationship as described by Johnson and Taylor (1978). The troponin concentration was determined according to Hartree (1972) using bovine serum albumin as a standard.

Labeling of Tropomyosins with *N*-[¹⁴C]Ethylmaleimide—Muscle, nonfusion and fusion tropomyosins were labeled at Cys¹⁹⁰ with *N*-[¹⁴C]ethylmaleimide in denaturing conditions as described by Heald and Hitchcock-DeGregori (1988).

Actin Binding Experiments—Binding assays of unlabeled and labeled tropomyosins to F-actin were carried out at 4 °C in a Beckman Airfuge for 30 min at 23 p.s.i. according to Heald and Hitchcock-DeGre-

gori (1988). The conditions of the experiments are described in the figure legends. Prior to mixing with actin, the tropomyosins were centrifuged in the Airfuge and the protein concentration determined. In experiments using unlabeled tropomyosin, actin-tropomyosin mixtures before and after centrifugation were analyzed by SDS-urea-polyacrylamide gel electrophoresis.

Viscosity Measurements—Viscosity measurements were carried out at room temperature ($26 \pm 1^\circ\text{C}$) using a Cannon-Manning semimicroviscometer (A 50) with a buffer outflow time of about 230 s. The experiments were carried out as described by Heeley *et al.* (1989).

Mg²⁺-ATPase Assays—The assay conditions are described in the figure legends. The samples were equilibrated at 25°C for 10 min before ATP (pH 7.0) was added. Inorganic phosphate was determined colorimetrically according to Heinonen and Lahti (1981). The incubation time was 10–30 min at 25°C , depending on the type of experiment. Control experiments demonstrated that the Mg²⁺-ATPase rate under these conditions was linear during the assay period (data not shown). The rate of ATP hydrolysis by myosin S1 was $0.05\text{--}0.10\text{ s}^{-1}$.

RESULTS

Characterization of Fusion Tropomyosins—Fig. 1C shows a Coomassie Blue-stained SDS-polyacrylamide gel of total bacterial extracts and pure samples of recombinant tropomyosins with different amino-terminal extensions. The structure of the amino terminus of these proteins is shown in Fig. 1B. These proteins were synthesized at high levels in a T7-based pET expression system with a final yield of 50 mg of pure protein/liter of induced culture.

As shown by Hitchcock-DeGregori and Heald (1987) and confirmed in this work, the primary sequence of the amino terminus of recombinant nonfusion tropomyosin is identical to the muscle protein except for the lack of acetylation at the amino-terminal methionine (Sodek *et al.*, 1978; Stone and Smillie, 1978). Amino-terminal sequence analysis of the recombinant fusion tropomyosins revealed that the initiation methionines of the fused peptides were removed, and therefore the purified fusion tropomyosins have amino-terminal fusions of 17 amino acids (S10FXTmy), 3 amino acids (AASmy), and 2 amino acids (ASTmy) (Fig. 1B). The sequence analysis of the proteins also shows no evidence of heterogeneity in the translation start site.

It is worth mentioning that the initiation methionines of recombinant S10FXTnI, TnC (Quaggio *et al.*, 1993) and the TnI deletion mutants TnI_{103–182} and TnI_{120–182} (Farah *et al.*, 1994) are removed. The amino-terminal sequences of these proteins and the fusion tropomyosins all begin with Met-Ala-Ser, which suggests that it might be a signal for the processing of first methionines in *E. coli*.

We compared the regulatory and physical properties of S10FXTmy, AASmy, ASTmy, and nFTmy with the chicken skeletal muscle α -tropomyosin (mTmy). We investigated their binding to actin, head-to-tail polymerization, their ability to inhibit the actomyosin Mg²⁺-ATPase and their ability to regulate the ATPase in a Ca²⁺-dependent manner in the presence of troponin.

Binding of Fusion Tropomyosins to F-actin—We analyzed the interaction of the different tropomyosins with F-actin by determining whether they cosediment with actin in a centrifugation assay. These experiments were performed using both unmodified and N-[C¹⁴]ethylmaleimide-labeled tropomyosins.

Binding of unmodified recombinant tropomyosins to F-actin was analyzed qualitatively by electrophoresis through SDS-urea-polyacrylamide gels. Fig. 2 shows mixtures before sedimentation and supernatants and pellets after cosedimentation. nFTmy was not detectable in the pellets after centrifugation (Fig. 2, lane 3), confirming the results of Hitchcock-DeGregori and Heald (1987) that recombinant nonfusion tropomyosin does not bind actin with high affinity. In contrast, all fusion tropomyosins were detectable in the pellets, demonstrating an effective binding to F-actin (Fig. 2, lanes 6, 9, and 12).

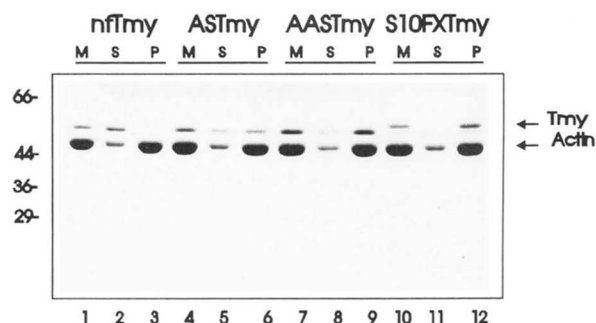


FIG. 2. **Actin binding of nonmodified tropomyosins.** Mixtures of actin and Tmy (M), supernatants (S), and pellets (P) from actin binding experiments are shown. Lanes 1–3, samples from nFTmy binding to actin; lanes 4–6, samples from ASTmy binding to actin; lanes 7–9, samples from AASmy binding to actin; lanes 10–12, samples from S10FXTmy binding to actin. Molecular masses of protein standards are indicated in kDa. Assay conditions: $5\text{ }\mu\text{M}$ actin and $1.0\text{ }\mu\text{M}$ tropomyosin were mixed in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl; 5 mM MgCl₂; 0.1 mM EGTA; 0.03% NaN₃ and 1.0 mM DTT. The binding of tropomyosins to F-actin was measured by cosedimentation as described under “Materials and Methods.” The samples were analyzed by 6 M urea, 1% SDS, 15% polyacrylamide gels to obtain a better separation of tropomyosins from actin. In the presence of urea, tropomyosin migrates anomalously with an apparent molecular weight of approximately 54,700 (Sender, 1971).

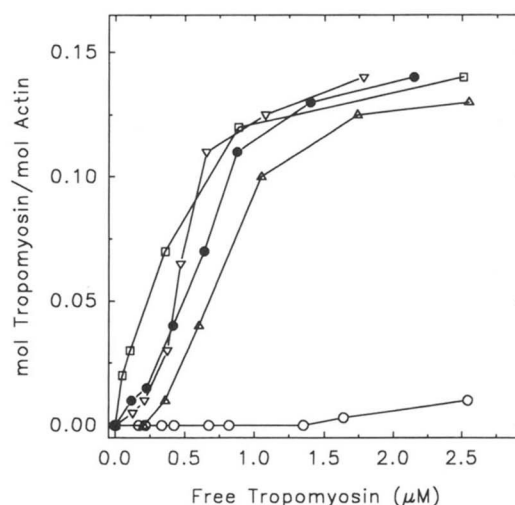
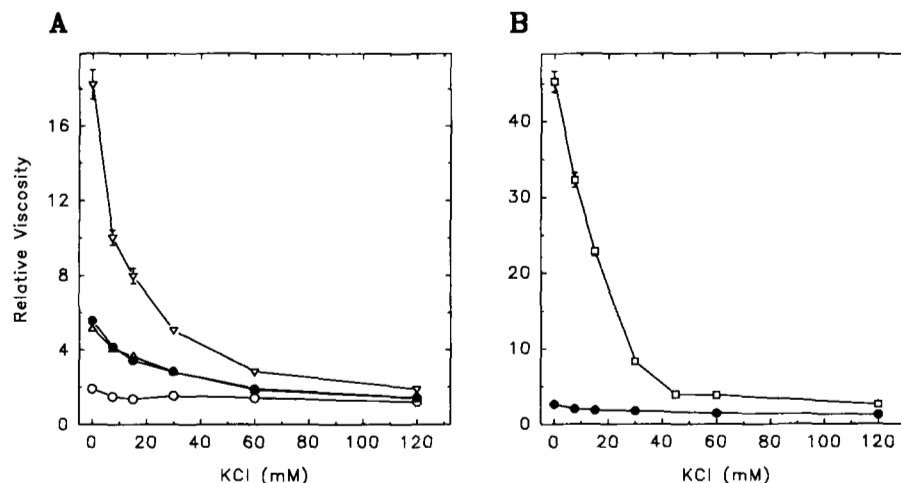


FIG. 3. **Binding of ¹⁴C-labeled tropomyosins to actin as a function of free tropomyosin.** The data are averages of two independent determinations. Assay conditions: $7\text{ }\mu\text{M}$ actin and ^{14}C -tropomyosin (from 0 to $3.45\text{ }\mu\text{M}$), in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.03% NaN₃, and 3 mM β -mercaptoethanol. The binding of labeled tropomyosins to actin was carried out as described under “Materials and Methods.” ●, mTmy; ○, nFTmy; △, ASTmy; ▽, AASmy; and □, S10FXTmy.

To obtain a quantitative measurement of the Tmy affinity for actin, we labeled the tropomyosins at Cys¹⁹⁰ with N-[¹⁴C]ethylmaleimide. nFTmy bound actin negligibly, too weak to obtain an accurate binding constant under the conditions of our assay (Fig. 3). These results are consistent with the results reported for nFTmy by Heald and Hitchcock-DeGregori (1988). Under the same conditions, all of our fusion tropomyosins bind to actin with binding isotherms similar to that of mTmy. The F-actin binding of all fusion tropomyosins and mTmy saturated at a molar ratio of tropomyosin:actin = 1:7.

The apparent binding constant of mTmy for actin was $1.6 \times 10^6\text{ M}^{-1}$, a value similar to that obtained by Yang *et al.* (1979), Mak *et al.* (1983), and Hill *et al.* (1992). ASTmy binds F-actin with a slightly lower affinity ($K_{\text{app}} 1.2 \times 10^6\text{ M}^{-1}$), whereas AASmy binds with a slightly higher affinity ($K_{\text{app}} 2.1 \times 10^6\text{ M}^{-1}$). S10FXTmy binds to actin with an affinity approximately

FIG. 4. Effects of ionic strength on tropomyosin polymerization. The determinations were done in triplicate, and the data are shown as the average $\pm$ standard deviations. Where an error bar does not appear, identical results were obtained in all three determinations. In *panel A* the protein concentrations were 2 mg/ml. In *panel B* the protein concentrations were 1 mg/ml because of the high viscosity of S10FXTmy. Assay conditions: 10 mM imidazole-HCl (pH 7.0), 20 mM DTT, and 0–120 mM KCl. The assays were done as described under "Materials and Methods." ●, mTmy; ○, nTmy; △, ASTmy; ▽, AASTmy; and □, S10FXTmy.



twice that of muscle Tmy (K_{app} 2.8×10^6 M $^{-1}$) but does not show a prominent cooperative behavior. The sigmoidal binding curves of AASTmy and ASTmy with actin are an indication of a cooperative process (Hill *et al.*, 1992).

Head-to-tail Polymerization of Fusion Tropomyosins—High ionic strength inhibits head-to-tail polymerization of Tmy and therefore reduces the viscosity of tropomyosin solutions (Tsao *et al.*, 1951). The relative viscosities of tropomyosins were measured as a function of salt concentration. nTmy does not polymerize (Fig. 4A and Hitchcock-DeGregori and Heald, 1987). In contrast, all of our fusion tropomyosins polymerize (Fig. 4, A and B). ASTmy and mTmy show identical salt dependence of polymerization, whereas AASTmy and S10FXTmy (Fig. 4B) have a higher propensity to head-to-tail polymerization. Because of the much larger viscosity of S10FXTmy solutions, the assays with this protein were performed using a lower concentration (1 mg/ml, Fig. 4B).

These results show that the introduction of 2 amino acids onto the amino terminus of recombinant tropomyosin restores its head-to-tail interaction to a level similar to that of mTmy.

Inhibition of S1 Mg $^{2+}$ -ATPase by Fusion Tropomyosins—In the absence of the troponin complex and at low molar ratios of myosin S1 to actin, mTmy inhibits the actomyosin S1 Mg $^{2+}$ -ATPase (Lehrer and Morris, 1982). The inhibitory properties of fusion tropomyosins were assessed by varying the concentrations of tropomyosin in the presence of a constant concentration of F-actin and S1 (Fig. 5).

nTmy has no effect on the actomyosin S1 Mg $^{2+}$ -ATPase (Fig. 5), a result reported previously by Cho *et al.* (1990). In contrast, all three fusion tropomyosins and mTmy demonstrate an inhibitory effect (Fig. 5). The inhibition obtained by ASTmy and AASTmy is very similar to the inhibition obtained with mTmy (Fig. 5). The maximum inhibition (approximately 70%) with mTmy, ASTmy, and AASTmy is observed at a molar ratio of Tmy:actin = 1:7 ([Tmy] = 0.8 μ M). In comparison, S10FXTmy, which binds strongly to actin, does not inhibit the actomyosin S1 Mg $^{2+}$ -ATPase as effectively (Fig. 5). The fact that the ATPase measurements must be performed at a much lower salt concentration, where S10FXTmy is partially polymerized (Fig. 4), precludes a direct comparison of these ATPase measurements with the actin binding experiments. Cho *et al.* (1990) reported that a tropomyosin with 80 amino acids fused to its amino terminus (NS1TM) inhibited as well as mTmy.

Our results demonstrate that two or three amino acid fusions to the amino terminus of recombinant tropomyosin recover this protein's ability to inhibit the actomyosin S1 Mg $^{2+}$ -ATPase.

Regulation of Mg $^{2+}$ -ATPase of Actomyosin S1 by Fusion Tropomyosins in the Presence of Troponin—The tropomyosin-tro-

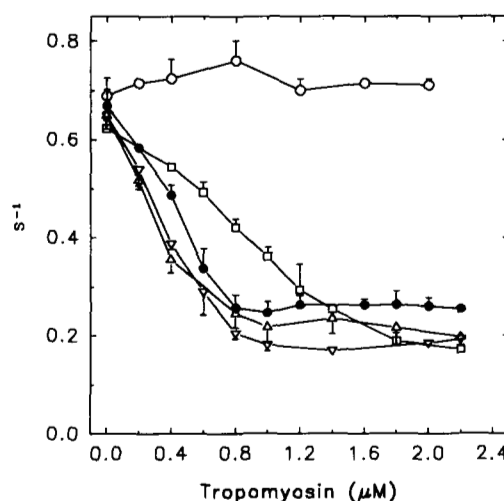


FIG. 5. Inhibition of actomyosin S1 Mg $^{2+}$ -ATPase by muscle, nonfusion, and fusion tropomyosins. Steady-state actomyosin Mg $^{2+}$ -ATPase was measured as a function of tropomyosin concentration. The data are the average $\pm$ standard error of four independent determinations at each Tmy concentration. Assay conditions: 0.5 μ M myosin S1, 5 μ M actin, 0–2.2 μ M tropomyosin in 5 mM imidazole-HCl (pH 7.0), 40 mM KCl, 0.5 mM DTT, 5 mM MgCl $_2$, 2 mM Na $_2$ ATP. ●, mTmy; ○, nTmy; △, ASTmy; ▽, AASTmy; and □, S10FXTmy.

ponin complex confers calcium sensitivity to the actomyosin Mg $^{2+}$ -ATPase. This complex has the ability to inhibit or potentiate the actomyosin ATPase in the absence and presence of calcium, respectively. To determine whether the fusion tropomyosins are capable of regulating the ATPase in a manner similar to mTmy, we determined the dependence of actomyosin ATPase in the presence of muscle troponin as a function of tropomyosin concentration (Fig. 6) and Ca $^{2+}$ concentration (Fig. 7).

As shown in Fig. 6, in the presence of troponin and calcium, the addition of mTmy potentiates Mg $^{2+}$ -ATPase activity. Maximum potentiation is achieved at a mTmy concentration of 0.8 μ M (where the molar ratio of tropomyosin to actin is approximately 1:7) (Fig. 6). At this molar ratio the ATPase activity is approximately three times that of the system without tropomyosin. At higher concentrations of mTmy the Mg $^{2+}$ -ATPase activity is inhibited. This effect of muscle α -tropomyosin was reported by Côté and Smillie (1981). In the absence of calcium, mTmy inhibits 50% of the actomyosin ATPase activity (Fig. 6). nTmy and the small fusion tropomyosins ASTmy and AASTmy show exactly the same concentration-dependent regulatory properties as mTmy (Fig. 6). In the presence of troponin in the

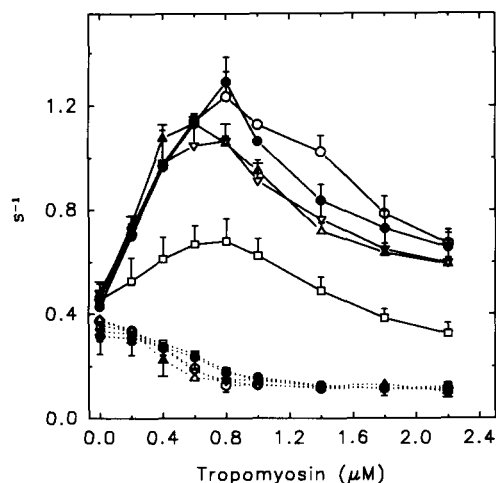


FIG. 6. Regulation of the actomyosin S1 Mg^{2+} -ATPase by muscle and recombinant tropomyosins (nonfusion and fusions) in the presence of troponin as a function of tropomyosin concentration. The data are average $\pm$ standard error of four independent determinations at each tropomyosin concentration. Assay conditions are the same as Fig. 5, with the following modifications: 2.2 μ M troponin, 0.5 mM $CaCl_2$ (solid lines), or 0.5 mM EGTA (dotted lines). $\bullet$, mTmy; $\circ$, nfTmy; $\triangle$, ASTmy; ∇ , AASTmy; and $\square$, S10FXTmy.

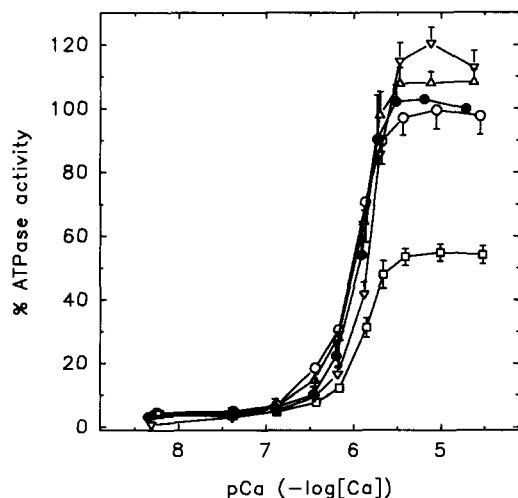


FIG. 7. Calcium regulation of the actomyosin S1 Mg^{2+} -ATPase by recombinant tropomyosins in the presence of troponin as a function of calcium concentration. The results are expressed as a percentage of the actin-activated Mg^{2+} -ATPase of myosin S1 obtained in the absence of troponin and tropomyosin. 0% is the activity of myosin S1 in the absence of actin-troponin and tropomyosin. The average $\pm$ standard error of three independent determinations for each pCa is shown. Assay conditions: 25 μ M actin, 7.14 μ M tropomyosin, 7.14 μ M troponin, and 0.3 μ M myosin S1 were combined in 20 mM imidazole-HCl (pH 7.0), 6.5 mM KCl, 3.5 mM $MgCl_2$, 1 mM DTT, 0.5 mM EGTA, 0.01% NaN_3 , 1 mM Na_2ATP , and $CaCl_2$ to give the free Ca^{2+} concentration indicated.

absence of calcium, the large fusion S10FXTmy inhibits the actomyosin S1 Mg^{2+} -ATPase as well as mTmy and the other recombinant tropomyosins (Fig. 6). However, in the presence of calcium, the activation obtained with S10FXTmy is less than the activation obtained with the other tropomyosins tested (Fig. 6).

A detailed analysis of the Ca^{2+} -dependent regulation of Mg^{2+} -ATPase by the different tropomyosins was obtained by determining the ATPase activity of the reconstituted thin filaments through a range of calcium concentration (Fig. 7). We observed that all of the recombinant tropomyosins can regulate the actomyosin S1 Mg^{2+} -ATPase activity as a function of calcium concentration. At $pCa \geq 7$ the actomyosin S1 Mg^{2+} -ATPase activity

is inhibited to the same extent by all tropomyosins. At $pCa \leq 5$, the ATPase activities of systems reconstituted with mTmy, nfTmy, ASTmy, and AASTmy were very similar and activated the ATPase to levels higher than that obtained with actin alone (Fig. 7). The ATPase rate of the reconstituted system with S10FXTmy at pCa 5.0 was approximately half that of the other tropomyosins tested.

All of these ATPase measurements clearly demonstrate that small fusions of 2 or 3 amino acids on the amino terminus of unacetylated tropomyosin do not interfere in its regulatory function on the actomyosin Mg^{2+} -ATPase. On the other hand, a larger fusion peptide of 17 amino acids diminished the Ca^{2+} activation of actomyosin Mg^{2+} -ATPase.

DISCUSSION

Our results show that the addition of 2 amino acids to the amino terminus of recombinant tropomyosin restores all functional properties known to depend on the acetylation of the initiation methionine present in mTmy.

The hydrophobic core positions of the heptad repeat (positions *a* and *d*) are important in the stabilization of the coiled coil structure of tropomyosin (Hodges *et al.*, 1981; Talbot and Hodges, 1982; Hodges, 1992). The initiation methionine occupies a core *a* position along the chain (Fig. 1B). In mTmy this residue follows the heptad repeat rule and has no net charge since the amino group is blocked, and the side chain is hydrophobic. In the recombinant molecule, the initiation methionine is not acetylated, and a net positive charge is present in this position. The two protonated amino groups from unacetylated tropomyosin are predicted to oppose each other in position *a* and *a'* in the hydrophobic core of the coiled coil. Electrostatic repulsions between these two groups may be expected to reduce the stability of the coiled coil in this region, thereby impairing actin binding and head-to-tail polymerization. Interactions that involve the 8–11 amino acid residues in the amino and carboxyl termini of tropomyosin are known to be responsible for these properties (Pato *et al.*, 1981; Mak and Smillie, 1981). A possible interpretation for our observations is that the fusion peptides remove the positive charge from the first methionine of unacetylated recombinant tropomyosin. The addition of 2 or 3 residues to recombinant tropomyosin moves the positively charged α -amino group from position *a* to positions *f* and *e*, respectively, thereby decreasing the electrostatic repulsion (Fig. 1B).

Cho *et al.* (1990) tested if the partial neutralization of the positive charge of the free α -amino group in nfTmy would be sufficient to restore actin binding. At the pH used (9.4), 60% or more of the amino termini of unacetylated tropomyosin would be deprotonated (Cho *et al.*, 1990). Actin binding was not restored under these conditions (Cho *et al.*, 1990). Based on these results it was postulated that the presence of a positive charge at the amino terminus in unacetylated tropomyosin is insufficient to explain the large difference in actin affinity between acetylated and unacetylated forms. Although the pK_a of the α -amino group of free methionine is 9.2 (Meisteir, 1965) it is known that the pK_a is influenced by the local environment, and one cannot be sure to what extent the amino terminus of the unacetylated tropomyosin molecule is deprotonated, unless the pK_a is determined experimentally. An alternative interpretation is that the unblocked amino terminus destabilizes the α -helix at the amino terminus of tropomyosin by interfering with the helix dipole (Fairman *et al.*, 1989; Shoemaker *et al.*, 1987; Chakrabarty *et al.*, 1991). The 2- or 3-amino acid extension would move the unstable region further upstream, stabilizing the helix and restoring function.

We do not know if the large difference in viscosity between S10FXTmy and AASTmy is caused by additional interactions

involving the extra amino acids in the S10 sequence. In any case it is clear that this longer fusion peptide disrupts the regulatory properties of tropomyosin. It has been demonstrated that longer fusions (80 amino acids present in NS1TM) impaired the head-to-tail association without affecting actin binding (Hitchcock-DeGregori and Heald, 1987).

The capacity to inhibit the actomyosin S1 Mg^{2+} -ATPase was also restored in the small fusions ASTmy and AASTmy. S10FXTmy showed a reduced inhibitory effect. In contrast, a larger fusion protein, NS1TM inhibited the Mg^{2+} -ATPase more effectively than muscle tropomyosin (Cho *et al.*, 1990).

In the presence of troponin, all of our recombinant tropomyosins regulate the actomyosin ATPase in a Ca^{2+} -dependent manner. nTmy, ASTmy, and AASTmy have regulatory properties very similar to mTmy. However, although a reconstituted thin filament containing S10FXTmy inhibited the ATPase in the absence of Ca^{2+} , a smaller activation of Mg^{2+} -ATPase was observed. This contrasts the properties of a larger fusion tropomyosin, NS1TM, with 80 amino acids fused to the amino terminus (Hitchcock-DeGregori and Heald, 1987). In the presence of troponin, NS1TM showed an impairment in its inhibitory activity (Hitchcock-DeGregori and Heald, 1987). These authors suggested that in the presence of troponin, NS1TM facilitates the switch of the thin filament to the "on" state in the presence of calcium but impairs the switch to the "off" state in the absence of calcium.

Our results demonstrate that the addition of 2 amino acids to the amino terminus of tropomyosin restores the functional properties that are known to depend on the acetyl group present in the muscle protein. We also demonstrate that this small fusion does not impair the functional properties affected by larger fusion peptides. By all criteria tested these mutant tropomyosins cannot be distinguished from mTmy.

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